University of Alberta

Mechanisms of Omega-3 Fatty Acid-Induced Suppression of Human Breast Cancer Cell Growth

By

©

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ABSTRACT

The omega-3 (or n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), inhibit the growth of human breast cancer cells in animal models and cell lines, but the mechanism by which this occurs is not well understood. The objective of this research was to identify the cellular mechanisms by which EPA and DHA modulate breast cancer cell growth. Human breast cancer cell lines (MDA-MB-231 and MCF-7) were chosen as the model to investigate direct effects of n-3 PUFA on tumor cell growth and death. A combination of EPA and DHA inhibited the growth of both cell lines by 45 to 50 % ($p<0.05$) in both the presence and absence of linoleic acid (LA), an essential omega-6 fatty acid. N-3 PUFA treatment decreased measures of tumor cell proliferation and increased measures of apoptotic cell death in both cell lines ($p<0.05$). EPA and DHA were significantly incorporated into cell membrane phospholipids of both cell lines treated with n-3 PUFA in both the absence and presence of LA ($p<0.05$). Cells incubated with n-3 PUFA demonstrated decreased Akt phosphorylation, as well as decreased NFκB DNA binding activity in the MDA-MB-231 cell line ($p<0.05$).

It was hypothesized that EPA and DHA would be incorporated into plasma membrane lipid rafts and this would affect aspects of signal transduction. Using a microscale method for the isolation of lipid rafts from mammary tumor cells that we adapted from existing protocols, it was confirmed that EPA and DHA were significantly incorporated into lipid rafts of both cell lines ($p<0.05$). Treatment with EPA and DHA (in the absence of LA) significantly decreased the sphingomyelin content and increased the phosphatidylcholine content of lipid rafts ($p<0.05$). N-3 PUFA treatment decreased the amount of epidermal growth factor receptor (EGFR) in rafts of MDA-MB-231 cells ($p<0.05$), which was accompanied by an increase in EGFR tyrosine phosphorylation.

In summary, the results of this research provide a greater understanding of how n-3 PUFA inhibit mammary tumor growth. This is important for the initiation of animal and human trials involving the use of dietary n-3 PUFA as adjuvants for cancer treatment.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate)
ANSA	8-anilino-1-naphthalene sulfonic acid
BCA	Bicinchoninic acid
BF_3	Boron trifluoride
BSA	Bovine serum albumin
°C	Degrees Celsius
Cdk	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
COX	Cyclooxygenase
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DMBA	Dimethylbenz(a)anthracene
DMSO	Dimethylsulfoxide
DPA	Docosapentaenoic acid
DPM	Decays per minute
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidylinositol
HRP	Horseradish peroxidase
IκB	Inhibitor of kappa B

IKK	Inhibitor of IκB kinase
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
LA	Linoleic acid
LDH	Lactate dehydrogenase
LNA	α-linolenic acid
LOX	Lipoxygenase
LTB_4	Leukotriene B_4
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MMP	Mitochondrial membrane potential
MUFA	Monounsaturated fatty acid
NFκB	Nuclear factor kappa B
NEU	N-nitroso-N-ethylurea
NMU	N-nitroso-N-methylurea
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E_2
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B (or Akt)
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PPAR	Peroxisome proliferator-activated receptor

PRK	Proliferation-related kinase
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error of the mean
SFA	Saturated fatty acid
SM	Sphingomyelin
TBS	Tris-buffered saline
TBST	Tris-buffered saline plus Tween-20
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TLC	Thin layer chromatography
TXA_2	Thromboxane A_2
UI	Unsaturation index
UV	Ultraviolet

1. INTRODUCTION AND LITERATURE REVIEW[1]

1.1 CANCER

Current incidence rates estimate that approximately 38 % of Canadian women and 43 % of Canadian men will develop some form of cancer (excluding basal cell and squamous cell carcinomas of the skin) during their lifetime (National Cancer Institute of Canada, 2004). Cancer is the uncontrolled growth of cells that results from defects in the regulatory circuits that govern normal cell proliferation. Cancer can arise in almost every tissue in the body, yielding more than 100 distinct types of cancer, and subtypes of tumors can be found even within specific organs (Weinberg, 1996; Hanahan and Weinberg, 2000). Cells in a tumor descend from a common ancestral cell that, over a long period of time, has accumulated a series of genetic mutations, each conferring one or another type of growth advantage leading to the conversion of normal human cells into cancer cells (Hanahan and Weinberg, 2000). The hallmarks of cancer include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Tumors become lethal when they invade and disrupt the tissues and organs needed for the survival of the organism as a whole (Weinberg, 1996).

1.1.1 Breast cancer

Breast cancer is the most frequently diagnosed cancer among women and is estimated to afflict 1 in 9 Canadian women in their lifetime (Canadian Breast Cancer Foundation, 2004). The causes of breast cancer are still largely unknown, but certain risk factors have been established. Increasing age is the most significant risk factor for breast cancer (Hankinson *et al.*, 2004). Risk also increases with the number of first-order relatives afflicted with the disease, such that family history is an important risk factor

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[1] A version of this chapter has been published in part. Field, C.J. & Schley, P.D. Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumor metabolism and immune function: lessons from n-3 fatty acids. *American Journal of Clinical Nutrition* 79:1190S-1198S, 2004.

(Hankinson *et al.*, 2004). A number of reproductive factors have also been linked to breast cancer risk, including early onset of menarche, late onset of natural menopause, late age at first full-term pregnancy, and nulliparity (Hankinson *et al.*, 2004; Pike *et al.*, 2004). These risk factors have implicated endogenous hormones, particularly estrogens, as underlying biological determinants of breast cancer incidence (Henderson *et al.*, 1988). Some inherited genetic mutations increase the risk of breast cancer (eg. mutations in the BRCA-1, BRCA-2, ATM and TP53 genes), however, such mutations are thought to account for only about 5 to 10 % of all breast cancers (Hodgson *et al.*, 2004). The role of dietary factors in the development or prevention of breast cancer has been highly controversial, and is still not well understood. The next sections of this literature review will focus primarily on the omega-3 fatty acids and their effects on breast cancer development.

1.2 OMEGA-3 (N-3) FATTY ACIDS AND BREAST CANCER

The omega-3 (or n-3) fatty acids refer to a class of polyunsaturated fatty acids (PUFA) having the first double bond in the n-3 position, or three carbons from the methyl end of the carbon chain (Figure 1-1). The n-3 fatty acids are considered essential as they cannot be synthesized by mammals and so must be obtained from the diet (Spector, 1999). The three main dietary n-3 fatty acids are α-linolenic acid (C18:3n-3, LNA), found in green leafy vegetables, walnuts, canola oil, soybean oil, and flaxseed, and the longer-chain eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), found primarily in cold-water fatty fish. Humans are able to produce EPA from LNA through a process of chain elongation and desaturation, however, the extent of this conversion is quite inefficient (~ 5-10 % of LNA is converted to EPA) (Jump, 2002; Davis and Kris-Etherton, 2003), such that EPA and DHA are acquired mainly through the consumption of fish. Amounts of n-3 fatty acids in fish vary widely depending on the type of fish and habitat in which they live, but in general, higher concentrations of EPA and DHA are found in sardines, salmon, mackerel, herring and rainbow trout (Larsson *et al.*, 2004). Unless otherwise stated, the use of 'n-3 PUFA' or 'n-3 fatty acid' in this thesis refers to the long-chain n-3 fatty acids (i.e. EPA and/or DHA).

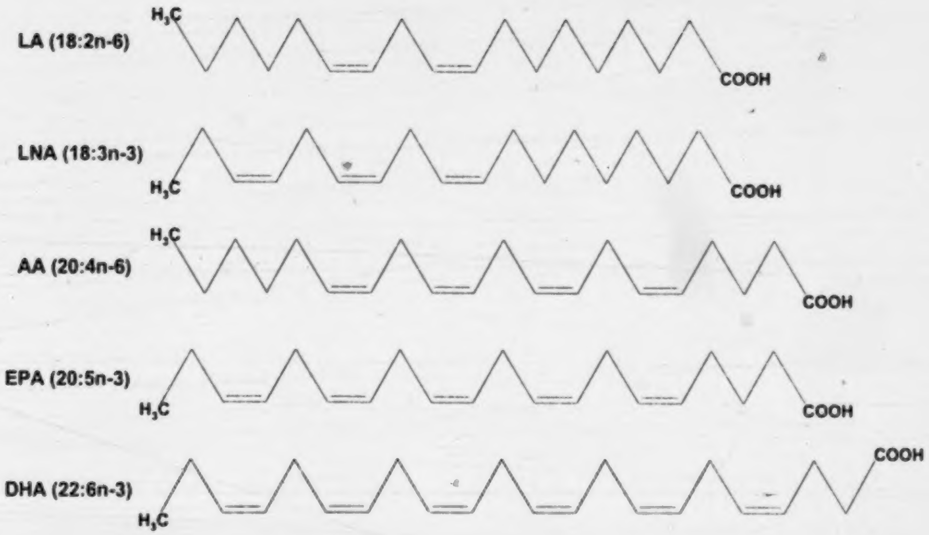

Figure 1-1 Structure and nomenclature of polyunsaturated fatty acids. The number before the colon indicates the number of carbons in the fatty acid chain and the number after the colon indicates the number of double bonds. The class of fatty acid is indicated by n-3 or n-6, which refers to the position of the first double bond at 3 or 6 carbons from the methyl end of the fatty acid chain, respectively. Abbreviations: LA, linoleic acid; LNA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

1.2.1 Epidemiological studies of n-3 fatty acid consumption and breast cancer risk

A potential role for n-3 fatty acids in the prevention of breast cancer development stemmed from observations that populations consuming large quantities of n-3 fatty acids from fish have low breast cancer rates (Kaizer *et al.*, 1989; Hursting *et al.*, 1990; Caygill *et al.*, 1996). In addition, migration studies demonstrated that Asian women who moved to North America and adopted a westernized diet experienced breast cancer rates similar to North American women, suggesting that environmental factors play a role in breast cancer risk (Ziegler *et al.*, 1993). While these studies may be confounded by other factors associated with a westernized lifestyle (eg. age at first pregnancy, duration of lactation), these ecologic studies generated the hypothesis that dietary n-3 fatty acids may protect against the development of breast cancer.

Analytic epidemiological studies having a case-control or cohort design have not supported a clear association between fish consumption and breast cancer risk (reviewed by de Deckere, 1999; Terry *et al.*, 2003; Terry *et al.*, 2004). While most of the studies have shown weak inverse associations between fish consumption and breast cancer risk, for the most part these associations have not reached statistical significance. In general, studies conducted in countries with relatively high consumption of fish (eg. Japan, Norway, Sweden, Finland) have found a 10 to 30 % lower risk of breast cancer with increased fish consumption, whereas studies conducted in North America (where fish intake is relatively low) have failed to find an association (Terry *et al.*, 2003). Thus, it is possible that in some populations the intake of long-chain n-3 PUFA is too low to produce a protective effect, or that the within-population variability of fish or n-3 fatty acid intake might be too low to detect differences in risk (Larsson *et al.*, 2004).

Inverse associations between marine fatty acid intake and breast cancer risk have been observed among studies that examined dietary intake of specific n-3 PUFA rather than total fish consumption (Terry *et al.*, 2003), perhaps highlighting the limitations of studies that only assessed total fish consumption (where actual n-3 fatty acid intake may vary widely depending on the type of fish consumed). Similarly, the absolute intake of fish may not be as predictive of breast cancer risk as is the intake of n-3 fatty acids relative to either n-6 or total fatty acids. This hypothesis is supported by an ecological study involving 24 European countries in which an inverse correlation was found between breast cancer risk and the ratio of fish or fish oil consumption to total or animal fat in the diet (Caygill *et al.*, 1996).

The epidemiological evidence for an inverse association between breast cancer risk and n-3 PUFA consumption is most compelling from studies that have examined biological markers of fish fatty acid consumption. These studies have found inverse associations between the levels of n-3 fatty acids, or the ratio of n-3 to n-6 fatty acids, in adipose tissue or serum phospholipids and breast cancer risk (Terry *et al.*, 2003; Terry *et al.*, 2004). A recent meta-analysis of biomarkers of dietary fat intake (i.e. fatty acid composition of blood or adipose tissue) and breast cancer risk found a significant protective effect of total n-3 PUFA against breast cancer risk from cohort (but not case-control) studies (Saadatian-Elahi *et al.*, 2004). These findings suggest that biological markers of n-3 PUFA consumption may more accurately reflect n-3 PUFA intake, and thus allow for more consistent observations of n-3 PUFA intake and breast cancer risk. Interestingly, two studies have shown that higher n-3 PUFA levels in breast adipose tissue are associated with better tumor response to cytotoxic drugs (Bougnoux *et al.*, 1994; Bougnoux *et al.*, 1999), indicating that n-3 PUFA in breast tissue may increase the efficacy of chemotherapeutic drugs and thus afford better patient prognosis.

More precise records of n-3 versus n-6 fatty acid intake, or measurements of biological markers of n-3 PUFA consumption, may help to elucidate whether n-3 fatty acid intake plays a role in breast cancer prevention in humans.

1.2.2 Animal studies of dietary n-3 fatty acids and breast cancer development

The inhibitory effects of n-3 fatty acids on the growth of mammary tumors in animals (primarily mice and rats) have been well documented (see Table 1-1). While there is limited evidence to suggest that n-3 PUFA influence cancer cell initiation (i.e. the induction of irreversible genetic damage), numerous studies suggest that n-3 PUFA can modulate tumor promotion (i.e. the selective outgrowth of initiated cells). This is supported by the great number of studies (see Table 1-1) that report a lower mammary tumor incidence, lower primary tumor weights or volumes, and lower proliferation rates of tumor cells in animals receiving either fish oil or purified long chain n-3 fatty acids in the diet. In addition, some studies report a reduction in the formation of metastases with dietary n-3 PUFA, suggesting that n-3 PUFA also modulate tumor progression (i.e. advancement to a highly malignant and invasive phenotype). N-3 PUFA effects are seen in a variety of tumor models, including carcinogen-induced, spontaneous, and transplantable mammary tumors. Anti-tumor effects have been observed with supplementation of n-3 fatty acids from as low as less than 1 % of the diet (by weight) from EPA or DHA, up to greater than 20 % of the diet (by weight) from fish oil. A meta-analysis of the relationship between dietary fats and mammary tumors in rodents showed a small protective effect of dietary n-3 PUFA on tumor development but the effect was not significant (Fay *et al.*, 1997). The majority of studies have examined the effects of the long-chain n-3 PUFA (i.e. EPA and DHA) on mammary tumor growth; however, anti-cancer effects are also documented with α-linolenic acid (see Table 1-1).

Table 1-1 Studies of dietary n-3 fatty acids and mammary tumor growth in animals

Reference	Animal model	Tumor	Total fat	N-3 PUFA content[1]	Results[2]	Mechanism tested
Karmali *et al*, 1984	Fischer 344 rats	R3230AC	5% w/w	FO (17, 33, or 67 mg EPA + 16, 32, or 64 mg DHA per d), fed 1 wk prior, 3 wks post-implant.	EPA/DHA ↓ tumor wt and vol vs. normal rat chow diet	EPA/DHA ↓ tumor eicosanoid (PGE_2, $PGF_{2\alpha}$, TXB_2) levels
Jurkowski and Cave, 1985	BUF rats	NMU-induced	0.5, 3 or 20% w/w	0.5, 3 or 20% w/w FO, fed 1 wk prior, 16 wks post-NMU	20% FO ↓ tumor incidence, tumor burden, and ↑ survival time vs. 0.5 or 20% CO or rat chow	N/D
Braden and Carroll, 1986	Sprague-Dawley rats	DMBA-induced	3, 10 or 20% w/w	3, 10, or 20% w/w FO (17% EPA, 9% DHA*), fed 1 wk post-DMBA until sacrifice	10 or 20% FO ↓ tumor incidence and multiplicity, and ↑ the latent period vs. 10 or 20% CO	N/D
Gabor and Abraham, 1986	BALB/c mice	IX	10% w/w	2.5, 5, 7.5, 9 or 10% w/w FO (16% EPA, 11% DHA), fed from d 1 to d 32 post-implant	9 or 10% FO ↓ tumor wt and vol vs. 10% CO	FO ↑ tumor cell loss (cell death)
Kort *et al*, 1987a	BN/Bi rats	BN472	30% en	9 or 23% en from FO (2 or 7% en from EPA+DHA), fed 9 wks prior, 17 d post-implant.	9 or 23% FO ↓ tumor size vs. cacao butter or sunflower oil diets	EPA/DHA ↓ plasma eicosanoid (PGE_2, TXB_2) levels
Kort *et al*, 1987b	BN/Bi rats	BN472	30% en	9 or 23% en from FO (2 or 7% en from EPA+DHA), fed 9 wks prior, 14 d post-implant.	no effect of EPA/DHA on lung metastases vs. cacao butter or sunflower oil diets	N/D
Abou-El-Ela *et al*, 1988	Sprague-Dawley rats	DMBA-induced	20% w/w	20% w/w FO (16% EPA, 11% DHA), fed from 3 to 16 wks post-DMBA	no sig. effect of FO on tumor incidence, burden, latency, or animal survival vs. 20% CO or PO	FO ↓ tumor eicosanoid (PGE; LTB_4, 6-keto-$PGF_{1\alpha}$) synthesis
Abou-El-Ela *et al*, 1989	Sprague-Dawley rats	DMBA-induced	20% w/w	15% w/w FO (16% EPA, 11% DHA), fed from 3 to 16 wks post-DMBA	15% FO ↓ tumor incidence vs. 20% CO	FO ↓ tumor eicosanoid (PGE, LTB_4) synthesis
Borgeson *et al*, 1989	BALB/c mice	MX-1	10% w/w	10% w/w FO (17% EPA, 11% DHA), fed 10 d prior, 5 wks post-implant.	10% FO ↓ tumor wt vs. 10% CO	FO altered tumor fatty acid metabolism
Kamano *et al*, 1989	SHN mice	spontaneous	10% w/w	10% w/w PO (58% LNA), fed from 2-3 to 13 months of age	10% PO ↓ tumor incidence, but not lung metastases vs. 10% SO or 10% soybean oil diets	N/D

Karmali *et al*, 1989	Sprague-Dawley rats	DMBA-induced	23.5% w/w	3, 8, or 20.5% w/w FO, or 23.5% BCO (16% LNA), fed 2 wks prior, 14 wks post-DMBA	all diets ↓ tumor incidence vs. 23.5% CO	N/D
Fritsche *et al*, 1990	BALB/c mice	410 or 410.4	10% w/w	10% w/w LO (56% LNA) or FO (16% EPA, 5% DHA), fed 3-10 wks prior and up to 13 wks post-implant.	LO ↓ tumor diameter and wt and ↑ survival time vs. 10% CO; neither diet ↓ tumor incidence, metastasis	tumor PGE_2 production was lowest with FO diet, but FO diet did not sig. affect tumor growth
Gabor *et al*, 1990	BALB/c mice	MX-1	11% w/w	11% w/w FO (18% EPA, 12% DHA), fed from day 5 to 30 post-implant	FO ↓ tumor vol vs 11% CO or lard	N/D
Takata *et al*, 1990	Sprague-Dawley rats	NMU-induced	5% w/w	4.7% w/w EPA, fed 1 wk prior, 20 wks post-NMU	EPA ↓ tumor incidence, wt, and multiplicity vs. 5% LA	EPA ↓ tumor eicosanoid (PGE_2, TXB_2, 6-keto-$PGF_{1\alpha}$) levels
Gonzalez *et al*, 1991	athymic nude mice	MCF-7, MDA-MB-231	5 or 20% w/w	19% w/w FO (10% EPA, 13% DHA), fed 7-10 d post-implant. for 6-8 wks	19% FO ↓ tumor vol but not incidence vs. 20% CO	FO alone ↑ tumor lipid peroxidation; vit E+FO ↓, oxidant+FO ↑, lipid perox. vit E+FO ↑, oxidant+FO ↓, tumor vol.
Wan *et al*, 1991	Fischer 344 rats	13762 MAT	20% w/w	20% w/w FO, fed 5 wks prior, 14 d post-implant	20% FO ↓ tumor vol and wt (n.s.) vs 20% SO	FO ↓ tumor and liver protein synthesis
Cohen *et al*, 1993	Fischer 344 rats	NMU-induced	23% w/w	5, 11.5 or 18% w/w FO (12.5% EPA, 11% DHA), fed from 2d post-NMU for 31 wks	only 11.5% FO ↓ total number of tumors per group vs. 23% CO	N/D
Craig-Schmidt *et al*, 1993	BALB/c mice	DMBA-induced	20% w/w	18% w/w FO (12% EPA, 5.5% DHA) fed 2 wks prior to DMBA until sacrifice	18% FO ↓ tumor incidence vs. 20% CO but not vs. 18% CCO; FO did not affect tumor multiplicity or survival	authors attribute the lower tumor incidence in FO and CCO groups to a lack of LA in diet
Gonzalez *et al*, 1993	athymic nude mice	MDA-MB-231	20% w/w	5, 10, 15 or 19 % w/w FO (16% EPA, 12% DHA), fed 7-10 d post-implant for 6-8 wks	all FO diets ↓ tumor vol vs. 20% CO	all FO diets ↑ tumor lipid peroxidation; vit. E + FO ↓ lipid perox. and ↑ tumor vol.
Karmali *et al*, 1993	Fischer 344 rats	13762MAT.B	23.5% w/w	23.5% w/w BCO (16% LNA), 15.5% BCO+8% FO (6% EPA, 3% DHA), or 20.5% FO (14% EPA, 8% DHA), fed 8 wks prior, 3 wks post-implant	all diets ↓ number and vol of lung metastatic foci vs. 23% CO	N/D
Rose and Connolly, 1993	athymic nude mice	MDA-MB-435	23% w/w	5, 11.5 or 18% w/w FO (17% EPA, 7% DHA), fed from wks 1 to 12 post-implant	11.5 and 18% FO ↓ tumor surface area, and 18% FO ↓ incidence and vol of lung metastases, vs. 5% FO	N/D

Welsch *et al*, 1993	athymic nude mice; beige-XID-athymic nude mice; SCID mice	MCF-7, MDA-MB-231	20% w/w	19% w/w FO (10% EPA, 13% DHA), fed 7-10 d post-implant. for 6-10 wks	FO ↓ tumor vol vs. 20% CO	authors conclude the mechanism does not involve immune cells (T, B, NK cells) in these immune-deficient mice
Rose *et al*, 1994	athymic nude mice	MDA-MB-435	23% w/w	5, 11.5 or 18% w/w FO (16% EPA, 12% DHA), fed from wks 1 to 12 post-implant	18% FO ↓ tumor surface area and incidence and vol of lung metastases vs 5% FO/18% CO	18% FO ↓ tumor PGE_2, but ↑ tumor LTB_4 and 5-HETE levels vs. 5%FO/18% CO; FO + vit E still sig. ↓ tumor surface area vs. 5% FO/18% CO
Williams and Maunder, 1994	Sprague-Dawley rats	NEU-induced	4% w/w	4% w/w FO, fed 20 wks post-NEU	FO ↓ tumor incidence and ↑ tumor latency vs. 4% CO or OO	N/D
Fernandes *et al*, 1995	MMTV/v-Ha-ras transgenic mice	spontaneous	20% w/w	20% w/w FO, fed from weaning to sacrifice (32 months of age)	FO ↓ tumor incidence (n.s.) but did not ↑ survival vs. 20% CO	N/D
Istfan *et al*, 1995	Fischer rats	13762 MAT	15% en	FO-enriched diet, fed 6 wks prior, 11 d post-implant.	FO ↓ tumor vol vs. 15% en SO	FO prolonged tumor cell S phase (DNA replication)
Liu and Rose, 1995	athymic nude mice	MDA-MB-435	20% w/w	4% w/w EPA, fed 7 d prior, 12 wks post-implant.	EPA ↓ incidence of lung metastases vs. 8% w/w LA	EPA ↓ mRNA expression of type IV collagenase in tumors
Munoz *et al*, 1995	BALB/c mice	M2, MM3	5% w/w	5% w/w mistol oil (22% LNA), fed 2 wks prior to implant until sacrifice	mistol oil ↑ tumor latency and survival time, and ↓ tumor vol vs. commercial diet control	N/D
Rose *et al*, 1995	athymic nude mice	MDA-MB-435	20% w/w	4 or 8% w/w EPA or DHA, fed 1 wk prior, 12 wks post-implant.	all diets ↓ tumor surface area, wt, and incidence of lung metastases vs. 8% w/w LA	EPA/DHA ↓ tumor eicosanoid (PGE, 12-HETE, 15-HETE) levels
Torosian *et al*, 1995	Lewis/Wistar rats	MAC-33	30% en	30% en FO (13% EPA, 10% DHA), or 15% FO/15% CO, fed from d 14 to d 35 post-implant.	30% FO and 30% CO ↓ tumor wt vs. FO/CO diet; FO/CO diet ↓ # of lung metastases vs. 30% CO	no diet affected tumor cell cycle distribution
Minami and Noguchi, 1996	Sprague-Dawley rats	DMBA-induced	0.5 or 20% w/w	0.5 mL EPA or DHA ethyl esters, fed twice per wk, from wks 1 to 20 post-DMBA	when given with the high fat diet, EPA and DHA ↓ tumor incidence and size vs. 0.5 mL CCO	EPA and DHA ↓ proliferation (BrdU labeling) of tumor cells

Rose *et al*, 1996	athymic nude mice	MDA-MB-435	20% w/w	2, 4, or 8% w/w EPA or DHA, fed 1 wk prior, and/or 8 wks post primary tumor excision	4 and 8% EPA and DHA ↓ pre-excision tumor surface area; 4 and 8% EPA ↓ pre-excision tumor wt; 8% EPA and DHA ↓ incidence and vol of lung metastases vs. 8% LA	N/D
Connolly *et al*, 1997	athymic nude mice	MDA-MB-435	23% w/w	18% w/w FO, fed 1 wk prior, 12 wks post-implant	FO ↓ tumor surface area and wt, and incidence, number and vol of lung metastases vs. 23% CO	FO ↓ tumor eicosanoid (PGE) levels
Noguchi *et al*, 1997	Sprague-Dawley rats	DMBA-induced	0.5 or 20% w/w	0.5 mL EPA or DHA ethyl esters, fed twice per wk, from wks 1 to 20 post-DMBA	when given with the high fat diet, EPA and DHA ↓ tumor incidence and multiplicity vs. 0.5 mL CCO	N/D
Hubbard *et al*, 1998	BALB/cAnN mice	4526	10 or 20% w/w	5, 9.5, 10 or 19% w/w FO, fed 4 wks prior to implant, until sacrifice	19% FO ↑ tumor latency and ↓ tumor vol vs. 20% SO, whereas 9.5 and 19% FO ↓ number and vol of lung metastases vs. 10 and 20% SO	N/D
Sasaki *et al*, 1998	Sprague-Dawley rats	DMBA-induced	10% w/w	6, 8, or 8.7% w/w FO (16% EPA, 12% DHA), fed 2 wks prior, 66 d post-DMBA	all FO diets ↑ tumor incidence, wt, and burden vs. mixed CCO-SO diet	all FO diets sig. ↓ tumor PGE_2 levels despite an ↑ in tumor growth
Senzaki *et al*, 1998	BALB/c mice	KPL-1	10% w/w	9.5% w/w EPA, fed 19 d prior, 43 d post-implant	EPA ↓ tumor incidence, wt, vol, and incidence of lymph node metastases vs. 10% LA or commercial diets	EPA ↓ tumor cell proliferation (Ki67 labeling) but did not ↑ apoptosis
Connolly *et al*, 1999	athymic nude mice	MDA-MB-231	20% w/w	2 or 4% w/w DHA, fed 1 wk prior, 12 wks post-implant.	4% DHA ↓ tumor surface area, and 2 or 4% DHA ↓ tumor wt vs. 8% LA	DHA ↓ tumor cell proliferation (Ki67 labeling), ↑ apoptosis (TUNEL labeling), and ↓ tumor eicosanoid (PGE_2, 12-HETE, 15-HETE) levels
Muñoz *et al*, 1999	BALB/c mice	M3	5% w/w	5% w/w mistol oil (22% LNA), fed 2 wks prior to implant until sacrifice	mistol oil ↓ tumor size, incidence of lung metastases and ↑ survival, but ↓ latency, vs. commercial diet (5% CO also ↓ tumor size and incidence of lung metastases)	N/D
Rose and Connolly, 1999	athymic nude mice	MDA-MB-231	20% w/w	4% w/w DHA, fed 1 wk prior, 12 wks post-implant.	4% DHA ↓ tumor wt vs. 4% LA	DHA ↓ angiogenesis (microvessel staining density) within tumors
Cognault *et al*, 2000	Sprague-Dawley rats	NMU-induced	15% w/w	15% w/w LO (59% LNA), fed from weaning to sacrifice	LNA ↑ tumor latency (n.s.) and ↓ tumor incidence (n.s.) vs. 15% mixed palm oil/sunflower oil	oxidant+LNA ↓ tumor multiplicity, area, and incidence, and ↑ tumor latency; vit E+LNA ↑ tumor area and multiplicity, and ↓ cell loss

Mukutmoni-Norris *et al*., 2000	BALB/cAnN mice	4526	10 or 20% w/w	5, 9.5, 10 or 19% w/w FO, fed 4 wks prior to implant, until sacrifice	no tumor growth data;	10, 20% FO ↓ tumor vascularization (blood vasculature area, mast cell #, endothelial staining) vs. 20% SO
Rao *et al*., 2000	TG.NK mice	MMTV/c-neu (spontaneous)	15% w/w	0.03, 0.06, or 0.11 g/d LNA, fed from 4 to 30 wks of age	0.11 g/d LNA ↓ tumor wt, burden and incidence (n.s.) vs. 0.22 g/d LA	N/D
Hardman *et al*., 2001	athymic nude mice	MDA-MB-231	5% w/w	3% w/w FO (34% EPA, 24% DHA), fed from wks 3 to 10 post-implant	FO ↓ tumor vol vs. 5% CO	FO ↑ tumor and liver lipid peroxidation
Robinson and Field, 2001	Fischer 344 rats	R3230AC	20% w/w	0.64% w/w EPA + 0.16% w/w DHA, fed 21 d prior, 17 d post-implant	EPA/DHA ↓ tumor wt (n.s.) vs. mixed SO/LO/beef tallow diet	EPA/DHA ↑ $CD8^+$ T cell activation and IFNγ and TNFα production
Chen *et al*., 2002	athymic nude mice	MDA-MB-435	20% w/w	10% w/w FS, fed from wks 8 to 15 post-implant	FS ↓ tumor surface area, vol (n.s.), wt (n.s.), and incidence of lung and lymph node metastases vs. 20% CO	FS ↓ tumor cell proliferation (Ki67 labeling) and IGF-I and EGFR protein staining in tumors
Yuri *et al*., 2003	Sprague-Dawley rats	NMU-induced	10% w/w	9.5% w/w EPA, 4.75% w/w EPA + 4.75% w/w DHA, or 9.5% w/w DHA, fed from 1 d post-NMU for 20 wks	DHA ↓ tumor incidence and multiplicity vs. EPA or EPA+DHA; no difference in latency among diets	N/D

Abbreviations: BCO, blackcurrant oil; CO, corn oil; CCO, coconut oil; DHA, docosahexaenoic acid; DMBA, 7,12-dimethylbenz(a)anthracene; en, energy; EPA, eicosapentaenoic acid; FO, fish oil, FS, flaxseed; GPX, glutathione peroxidase; HETE, hydroxyeicosatetraenoic acid; IFN, interferon; LA, linoleic acid, LNA, alpha linolenic acid; LO, linseed oil; NEU, N-nitroso-N-ethylurea; NMU, N-nitroso-N-methylurea; N/D, not determined; n.s., not significant; OO, olive oil; PO, perilla oil; SO, safflower oil; TNF, tumor necrosis factor; vol, volume; wt, weight;

[1] when available, the composition of n-3 PUFA-containing oil is given; values are the percentage of total fatty acids in the oil

[2] tumor incidence = number of animals with tumors/total number of animals; tumor multiplicity = # of tumors/tumor bearing animal; tumor burden = weight of tumors/tumor bearing animal; tumor latency = time until appearance of the first tumor

1.2.3 Cell culture studies of n-3 fatty acid exposure and breast cancer cell growth

The literature supporting the effects of n-3 fatty acids on human breast cancer cell lines *in vitro* is not as extensive as the *in vivo* animal studies, but the results of the cell culture studies are consistent with the animal studies and the human epidemiological diet-cancer incidence associations. *In vitro* experiments have demonstrated that n-3 fatty acids, particularly DHA and EPA, decrease the growth (i.e. the number of cells present after fatty acid exposure versus the number of untreated or control-treated cells) of different human breast cancer cell lines including MDA-MB-231, MCF-7, KPL-1, ZR-75-1, and T47-D (see Table 1-2 for a review of these studies). Growth inhibition was seen in these cell lines with fatty acid concentrations ranging from less than 1 μg/ml (approx. 3 μM DHA and 3.3 μM EPA) to greater than 600 μM of purified fatty acid. Discrepancies in the inhibitory fatty acid concentrations used among studies are likely related to differences in culture conditions (eg. concentration of fetal calf serum, form of fatty acid delivered to the cells, initial cell seeding density, cell line growth characteristics), as these varied widely from study to study, and are likely to have significant effects on cell growth. For example, in many of the above studies, fatty acids were added to the culture media as free fatty acids dissolved in ethanol (i.e. not bound to protein such as albumin), a form that has direct cytotoxic effects on cells (Rose and Connolly, 1990). The concentration of FCS used in the media, as well as cell seeding density, also affects the toxicity of n-3 PUFA, as a protective effect of increasing FCS concentration and cell seeding density on n-3 PUFA-induced growth inhibition has been reported (Begin *et al.*, 1985; Begin *et al.*, 1986).

Table 1-2 Studies of n-3 fatty acids and mammary tumor cell growth *in vitro*

Reference	Cell line	N-3 fatty acid	Concentration[1]	Culture conditions	Results	Mechanism tested
Begin *et al*, 1985	ZR-75-1	LNA and DHA ethyl esters, EPA methyl ester	20 µg/ml	10% FCS, no BSA	all n-3 FAs ↓ the number of viable cells vs. untreated[2] cells	all n-3 FAs ↑ the number of non-viable cells; vit. E abrogated the FA effects
Begin *et al*, 1986	ZR-75-1	LNA and DHA ethyl esters, EPA methyl ester	20 µg/ml	10% FCS, no BSA	all n-3 FAs ↓ the number of viable cells vs. untreated cells	EPA>LNA>DHA ↑ the number of non-viable cells
Begin *et al*, 1988	ZR-75-1	DHA ethyl ester, EPA methyl ester	20 µg/ml	10% FCS, no BSA	EPA > DHA ↑ the number of non-viable cells vs. untreated cells	EPA > DHA ↑ lipid peroxidation
Rose and Connolly, 1990	MDA-MB-231	DHA, EPA	0.25, 0.5, 0.75, 1.0, 1.5, 2.0, or 2.5 µg/ml	no FCS, 1.25 mg/ml BSA; 10 µg/ml insulin	DHA (≥ 1.5 µg/ml) and EPA (2.5 µg/ml) ↓ the number of viable cells vs. untreated cells	neither FA ↑ the number of non-viable cells
Begin and Ells, 1992	ZR-75-1	DHA ethyl ester	20 µg/ml	10% FCS, no BSA	DHA ↑ the number of non-viable cells vs. untreated cells	DHA ↑ lipid peroxidation
Grammatikos *et al*, 1994	MCF-7	LNA, DHA, EPA	6, 12, 18, 24, or 30 µM	0.5% FCS, 1.5 mg/ml BSA	EPA, DHA > LNA ↓ the number of viable cells in a dose-dependent manner vs. OA-treated cells	no FA ↑ the number of non-viable cells or lipid peroxidation
Chajes *et al*, 1995	MCF-7, ZR-75, T-47D, HBL-100, MDA-MB-231	LNA, EPA, DHA methyl esters	20 µg/ml	5% FCS, no BSA	all n-3 FAs ↓ the number of viable ZR-75, T-47D, HBL-100, and MDA-MB-231 cells, whereas only DHA ↓ the number of viable MCF-7 cells, vs. untreated cells	no FA ↑ the number of non-viable cells, but all FAs ↑ lipid peroxidation; vit. E abrogated the FA effects
Noguchi *et al*, 1995	MDA-MB-231	EPA, DHA	0.1, 0.3, 0.9, 2.7, or 8.1 µg/ml	no FCS, 1.25 mg/ml BSA; 10 µg/ml insulin, 625 ng/ml LA	both FAs ↓ the number of viable cells at 0.9-8.1 µg/ml, and thymidine incorporation at 8.1 µg/ml only, vs. untreated cells	both FAs ↓ PGE and LTB secretion
Bardon *et al*, 1996	T47D	LNA	2.5 - 25 µg/ml	1% FCS, 1 mg/ml BSA, 5 µg/ml insulin	LNA did not affect the numbers of viable or non-viable cells vs. untreated cells	N/D

Abdi-Dezfuli *et al*., 1997	MCF-7	EPA, DHA	4-64 μM	serum-free, no BSA, 50 nM insulin, 0.5 nM estradiol	EPA at ≥ 32 μm ↓ the number of cells, whereas DHA had no effect, vs. untreated cells	N/D
Katdare *et al*., 1997	MMEC initiated with carcinogens and oncogenes	EPA	16 μM	10% FCS, no BSA, 5 μg/ml insulin	EPA ↓ the number of anchorage-independent cell colonies formed in culture vs. untreated cells	N/D
Telang *et al*., 1997	MMEC initiated with carcinogens and oncogenes	EPA	16 μM	10% FCS, no BSA, 5 μg/ml insulin	EPA ↓ the number of anchorage-independent cell colonies formed in culture vs. untreated cells	EPA ↓ Ras p21-GTP binding and estradiol metabolism
Senzaki *et al*., 1998	KPL-1	EPA	10^{-2} - 100 μg/ml	10% FCS, no BSA	EPA ↓ the number of viable cells at > 5 μg/ml vs. untreated cells	N/D
Yamamoto *et al*., 1999	MDA-MB-231, T-47D, MCF-7, KPL-1, MKL-F	EPA ethyl ester	10^{-4} - 400 μg/ml	10% FCS, no BSA	EPA > 1 μg/ml ↓ the number of viable cells in all cell lines vs. untreated cells (IC_{50} = 69-221 μg/ml^{3})	EPA ↑ apoptosis, ↓ bcl-2 and ↑ bax protein levels, and arrested cell cycle in KPL-1
Nakagawa *et al*., 2000	MCF-7, MDA-MB-231	EPA	3.2 nM - 1.28 mM	10% FCS, no BSA	EPA ↓ the number of viable cells in both cell lines vs. untreated cells (EPA IC_{50} = 620.2 μM in MCF-7, and 454.7 μM in MDA-MB-231)	N/D
Wang *et al*., 2000	MDA-MB-231	DHA	2, 4, 6, 8, or 12 μg/ml	1% FCS, no BSA	8 and 12 μg/ml DHA ↓ the number of viable cells vs. untreated cells	N/D
Kachhap *et al*., 2001	MCF-7	DHA	1.5 μg/ml	10% FCS, BSA	DHA ↓ thymidine incorporation vs. untreated cells	DHA caused cell cycle arrest, but no apoptosis
Moore *et al*., 2001	MCF-7, MDA-MB-231	EPA, DHA	5 μg/ml	1% FCS, 1.25 mg/ml BSA	no growth (eg. cell count) data	in MDA-MB-231, EPA and LA ↓ PKA activity vs. untreated cells; EPA > DHA >>LA ↓ PKA RIα and PKCα expression
Bernard-Gallon *et al*., 2002	MCF-7, MDA-MB-231	EPA methyl ester DHA methyl ester	1.5 - 300 μM	2% FCS, no BSA	EPA and DHA ↓ the number of viable cells vs. untreated cells (EPA IC_{50} = 9, 30 μM, DHA IC_{50} = 3, 30 μM (MDA-MB-231, MCF-7))	EPA and DHA ↑ BRCA1/2 mRNA but not protein levels

Chamras *et al*., 2002	MCF-7	EPA methyl ester DHA methyl ester	100 μM	5% FCS, no BSA	EPA and DHA ↓ the number of viable cells, and anchorage-independent colonies formed in culture, vs. untreated cells	neither FA ↑ the number of non-viable or apoptotic cells, or altered cell cycle distribution; vit E did not abrogate the FA effects; both FAs ↑ differentiation (lipid droplet accumulation)
Pasqualini *et al*., 2003	MCF-7	EPA	50 μM	10% FCS, no BSA	EPA ↓ the number of viable cells vs. untreated cells	EPA ↑ E-cadherin protein expression
Baumgartner *et al*., 2004	MCF-7, SK-BR-3, MDA-MB-468	DHA	25 - 100 μM	10% FCS, no BSA	100 μM DHA ↓ the number of viable cells of all cell lines vs. untreated cells	N/D
Menendez *et al*., 2004	SK-BR-3	ALA, EPA, DHA methyl esters	6.25 - 50 μg/ml	10% FCS, no BSA	LNA > DHA > EPA ↓ the number of viable cells vs. untreated cells	LNA > DHA > EPA ↓ FAS activity; vit E abrogated the FA effects on FAS
Tsujita-Kyutoku *et al*., 2004	KPL-1	DHA ethyl ester	1 - 500 μM	10% FCS, no BSA	DHA ↓ the number of viable cells vs. untreated cells (IC_{50} = 270 μM)	DHA ↑ apoptosis, arrested cell cycle, ↑ p53 and p21 and ↓ cyclin D1 and bcl-2 protein levels
Utomo *et al*., 2004	MCF-7, HCC1937	EPA	0.1 - 10 μg/ml	1% FCS, no BSA	EPA ≥ 0.5 μg/ml ↓ the number of viable cells of both cell lines vs. untreated cells	↑ the expression of a novel phospholipid GPX in both cell lines abrogated the effects of EPA, implicating lipid peroxidation

Abbreviations: BSA, bovine serum albumin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAS, fatty acid synthase; FCS, fetal calf serum; GPX, glutathione peroxidase; LNA, α-linolenic acid; MMEC, mouse mammary epithelial cells; N/D, not determined; OA, oleic acid; PKA, protein kinase A; PKC, protein kinase C;

[1] 1 μg/ml EPA = 3.3 μM; 1 μg/ml DHA = 3 μM; 1 μg/ml LNA = 3.6 μM

[2] untreated cells refers to control cultures grown in parallel with the fatty-acid treated cells but not exposed to exogenous fatty acids

[3] IC_{50} = the concentration required to inhibit growth (i.e. reduce the number of viable cells) by 50 %

1.3 POTENTIAL MECHANISMS OF N-3 FATTY ACID-MEDIATED SUPPRESSION OF BREAST CANCER CELL GROWTH

A large number of studies demonstrate growth-inhibitory effects of n-3 PUFA on mammary tumor cells in animals and in culture. Many mechanisms have been proposed to explain the anti-cancer effects of n-3 fatty acids. The remaining sections of this literature review will describe the potential mechanisms by which n-3 PUFA may inhibit mammary tumor cell growth.

1.3.1 Cell proliferation versus cell death

N-3 fatty acids may hinder the growth (i.e. clonal expansion, or increase in cell number) of tumor cells through an impairment of cell proliferation (eg. by decreasing growth-stimulatory signal transmission), or an increase in cell death (eg. by promoting death signaling), or a combination of both. These two outcomes are not necessarily mutually exclusive, since many signaling pathways can mediate both cell survival and cell death (eg. growth factor withdrawal can decrease cell proliferation and induce apoptotic cell death; Okada and Mak, 2004). The next sections of this literature review will summarize the evidence for the effects of n-3 PUFA on cell proliferation and cell death, and will then examine possible mechanisms by which these outcomes occur.

1.3.1.1 Cellular replication

Cellular replication, or the cell cycle, is essentially composed of four distinct phases (Figure 1-2): G_1 is an initial growth phase that leads to DNA synthesis (S phase), followed by a gap phase (G_2), and finally by mitosis (M phase), the actual segregation of chromosomes and cytoplasms (Leake, 1996). Cells may also exit the cell cycle into G_0 phase, a sustained resting phase that is characteristic of many terminally differentiated cells. Normal cells progress through the cell cycle after stimulation by exogenous agents such as growth factors, hormones, or cytokines (Leake, 1996). Cancerous cells, however, appear to lose their dependency upon these external signals and often progress, unregulated, through many cell cycles (Hanahan and Weinberg, 2000). Multiple specific mutations in the genes encoding proteins that regulate cell cycle progression have been identified in tumor cells (Dictor *et al.*, 1999).

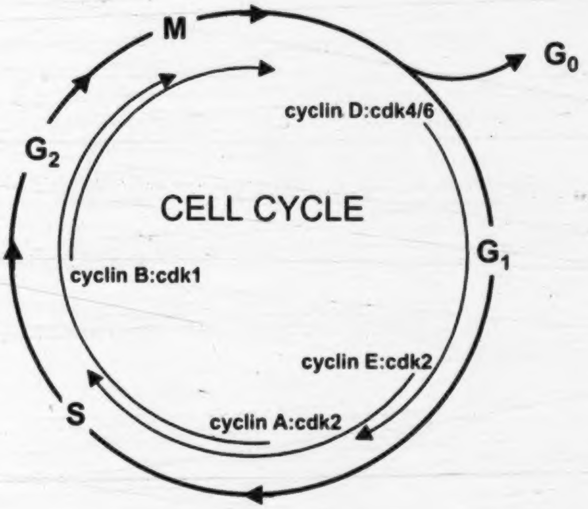

Figure 1-2 The cell cycle

Two important families of regulatory molecules promote progression through the cell cycle, the cyclins and the cyclin-dependent kinases (cdks). Cyclins are proteins whose levels oscillate throughout the cell cycle. Cyclins bind to and activate cdk molecules, such that the cell cycle is regulated primarily by the sequential activation and inactivation of cdks through the periodic synthesis and destruction of cyclins. The D-type cyclins (eg. cyclins D1, D2, D3) regulate the progression of cells through G1, and their levels are controlled by exogenous growth factors. The D-type cyclins bind and activate cdk4 and cdk6, leading to the activation of transcription factors that transcribe genes required for cell cycle progression and DNA synthesis. Cyclin E is induced during the progression of cells through G1, and is required for transition from G1 into S phase. Cyclin A accumulates through S phase, and is required for completion of S phase and entry into M phase (Johnson and Walker, 1999).

Entry into, and exit from, mitosis is regulated by the B-type cyclins (cyclins B1, B2) in association with cdk1. Activation of the cyclin B/cdk1 complex depends on both the levels of cyclin B as well as the phosphorylation status of cdk1. Neither the signals nor the transcription factors that induce expression of cyclin B1 have been conclusively identified, although the NF-Y and USF-1 transcription factors have been implicated (Cogswell *et al.*, 1995; Katula *et al.*, 1997; Cheung *et al.*, 1999; Farina *et al.*, 1999). Activated cyclin B/cdk1 phosphorylates cytoskeleton proteins as well as components of the mitotic spindle, allowing for reorganization of the subcellular architecture, and ultimately cell division (Pines, 1998). Exit from mitosis requires the destruction of cyclin B, which occurs through polyubiquitination and proteosomal degradation.

Negative regulators of the cell cycle include the retinoblastoma protein (Rb), the p53 transcription factor, and the family of cdk inhibitors (CDKIs) consisting of $p21^{WAF1/CIP1}$, p27, p57, and the INK4 proteins. The Rb protein inhibits cell proliferation in all tissues (Leake, 1996). In its dephosphorylated state, it binds and inactivates proteins including transcription factors that are involved in cell cycle progression and DNA synthesis. Rb is phosphorylated by the D-type cyclins, resulting in its dissociation from, and activation of, its target proteins. P53 is a transcription factor that is stabilized and activated in response to DNA damage (Johnson and Walker, 1999). P53 can block progression through S phase by inducing the expression of genes involved in growth arrest and repressing the expression of genes involved in growth promotion (Leake, 1996). If the DNA is damaged beyond repair, p53 activation can lead to apoptotic cell death (Shaw, 1996). P53 also induces the expression of members of the CDKI family, namely $p21^{WAF1/CIP1}$. The CDKIs inhibit cell cycle progression by binding to and inhibiting the kinase activity of cyclin/cdk complexes.

1.3.1.2 Effects of n-3 fatty acids on cell proliferation

Only a few studies have examined the effects of n-3 PUFA on proliferation of breast cancer cells. Among the *in vitro* reports, EPA and DHA arrested cell cycle progression in KPL-1 cells (Yamamoto *et al.*, 1999; Tsujita-Kyutoku *et al.*, 2004), while DHA was reported to induce a cell cycle arrest of MCF-7 cells (Kachhap *et al.*, 2001). One study found no effect of either EPA or DHA on the cell cycle distribution of MCF-7 cells (Chamras *et al.*, 2002). Among the animal studies, prolonged DNA synthesis in mammary tumor cells was observed with dietary fish oil (Istfan *et al.*, 1995), while three studies found that EPA and/or DHA feeding decreased indices of proliferation (eg. BrdU labeling, Ki67 staining) in mammary tumor cells (Minami and Noguchi, 1996; Senzaki *et al.*, 1998; Connolly *et al.*, 1999). Conversely, Torosian *et al.* (1995) found no effect of fish oil feeding on mammary tumor cell cycle distribution.

Among other tumor types, DHA has been shown to arrest progression through the cell cycle in malignant melanoma cells *in vitro* (Albino *et al.*, 2000). Similarly, EPA treatment *in vitro* is reported to arrest the growth of K-562 human leukemic (Chiu *et al.*, 2001), pancreatic (Lai *et al.*, 1996), and colon (Clarke *et al.*, 1999) cancer cell lines in different phases of the cell cycle, correlating with a downregulation of cyclin protein expression (cyclins E and B1) in one instance (Chiu *et al.*, 2001).

1.3.1.3 Cell death

Until recently, cell death was believed to occur by one of two mechanisms: 1) necrosis, which often results from some sort of insult or toxicity, and triggers inflammation, or 2) apoptosis, the energy-requiring process of programmed cell death, or cell suicide. Necrotic cells are characterized by ATP-depletion leading to plasma membrane blebs, cytoskeletal alterations and loss of volume control, mitochondrial permeabilization, cell swelling, and leakage of small molecules, eventually leading to a fatal rupturing of the plasma membrane. Necrotic cells release a variety of cytosolic enzymes including lactate dehydrogenase and aminotransferases into the extracellular medium, and will also take up supravital dyes like trypan blue or propidium iodide that are excluded by viable cells (Kim *et al.*, 2003). In contrast, apoptosis is an ATP-requiring process that is tightly regulated by a variety of signals, and is characterized by a number of identifiable features, including cell shrinkage, loss of mitochondrial membrane potential, DNA fragmentation, chromatin condensation, and nuclear membrane fragmentation. Apoptosis is mediated by a family of cysteine proteases known as the caspases, which are activated specifically in apoptotic cells (Okada and Mak, 2004). Caspases are initially expressed as inactive caspase precursors. When initiator caspases, such as caspase-8 and caspase-9, are activated by apoptotic stimuli, they cleave the precursor forms of the effector caspases (eg. caspase-3, caspase-6, caspase-7). These effector caspases cleave a restricted set of target proteins, leading to the biochemical and morphological features of apoptosis described above (Okada and Mak, 2004). In classic apoptotic cell death, cells fragment into apoptotic bodies, and intracellular contents are not released, so inflammation is not triggered *in vivo* (Kim *et al.*, 2003).

Recent studies indicate that necrosis and apoptosis are not distinct and independent entities (reviewed by Kim *et al.*, 2003; Jaeschke and Lemasters, 2003). The mode of cell death occurring in a cell appears to depend largely on the ATP supply of the cell. Cellular levels of ATP can mediate a switch from apoptosis to necrosis or vice versa. When ATP is depleted, apoptosis is blocked, and proapoptotic signals can induce necrotic cell death (Kim *et al.*, 2003). Even after initiation of apoptosis, necrosis may supervene if ATP levels subsequently fall, or if another change leads to a breakdown of the plasma membrane barrier. On the other hand, partial ATP recovery can prevent necrotic cell death, with apoptosis developing instead as a result of caspase activation by cytochrome c released from mitochondria with a loss of membrane potential. Thus, it is more likely that apoptosis and necrosis represent extremes on a continuum, where cells undergo modes of cell death that can display both apoptotic and necrotic patterns of cellular damage (Kim *et al.*, 2003). This paradigm has been termed "necrapoptosis" (Kim *et al.*, 2003; Jaeschke and Lemasters, 2003).

1.3.1.4 Effects of n-3 fatty acids on cell death

Early studies of n-3 PUFA and breast cancer cell growth *in vitro* attributed the growth-inhibitory effects of EPA and DHA to cytotoxic (necrotic) cell death (Begin *et al.*, 1985; Begin *et al.*, 1986; Begin *et al.*, 1988). This was concluded to be the result of an increased rate of lipid peroxidation. Alternatively, this cytotoxic effect of PUFA may have simply been the result of free fatty acids in the media, as the methodology did not allow for any binding of fatty acids to protein, such as BSA. Subsequent studies that provided fatty acids complexed with BSA did not report an increase in non-viable cells with fatty acid exposure (Rose and Connolly, 1990; Grammatikos *et al.*, 1994). Among the animal studies, decreased mammary tumor growth has been attributed to "tumor cell loss" (Gabor and Abraham, 1986), although the manner of cell death was not discussed.

There is some evidence for n-3 fatty acids acting to increase apoptosis in certain cell types, but the evidence to date is not strong, especially in breast cancer cells. Only a few studies have documented n-3 PUFA-induced apoptosis in breast cancer cells. *In* *vitro*, EPA and DHA induced apoptosis of KPL-1 breast cancer cells (Yamamoto *et al.*, 1999; Tsujita-Kyutoku *et al.*, 2004), however two studies found no effect of EPA or DHA on apoptosis in MCF-7 cells (Kachhap *et al.*, 2001; Chamras *et al.*, 2002). *In vivo*, DHA provided in the diet to mice induced apoptosis and partial inhibition of proliferation of implanted MDA-MB-231 cells (Connolly *et al.*, 1999), whereas EPA feeding did not induce apoptosis in KPL-1 cells (Senzaki *et al.*, 1998). While more studies are clearly needed to establish the effects of n-3 PUFA on apoptosis in breast cancer cells, n-3 PUFA have been shown to induce apoptosis in other transformed cells, including colon cancer cells (Clarke *et al.*, 1999; Chen and Istfan, 2000; Narayanan *et al.*, 2001; Latham *et al.*, 2001), lymphoma cells (Heimli *et al.*, 2001), leukemic cells (Chiu and Wan, 1999; Chiu *et al.*, 2000), hepatocarcinoma cells (Calviello *et al.*, 1998) and pancreatic cancer cells (Lai *et al.*, 1996; Hawkins *et al.*, 1998).

1.3.2 Mechanisms by which n-3 PUFA alter tumor cell proliferation or death

The proposed mechanisms by which n-3 PUFA may alter tumor cell proliferation or death are shown schematically in Figure 1-3, and described in detail below.

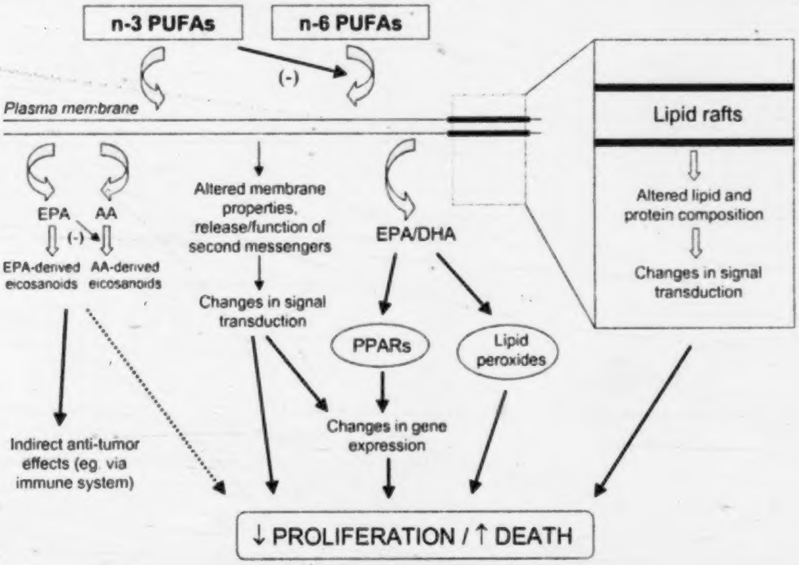
n-3 PUFAs
n-6 PUFAs
(-)
Plasma membrane
Lipid rafts
Altered lipid and protein composition
Changes in signal transduction
EPA
AA
(-)
EPA-derived eicosanoids
AA-derived eicosanoids
Altered membrane properties, release/function of second messengers
Changes in signal transduction
EPA/DHA
PPARs
Lipid peroxides
Changes in gene expression
Indirect anti-tumor effects (eg. via immune system)
↓ PROLIFERATION / ↑ DEATH

Figure 1-3 Possible mechanisms of n-3 PUFA-induced inhibition of mammary tumor cell growth.

1.3.2.1 N-3 PUFA may interfere with eicosanoid production from arachidonic acid

The n-6 and n-3 classes of fatty acids are incorporated into the phospholipids of cell membranes of many cell types, including tumor cells, following n-6 or n-3 PUFA inclusion in the diet or cell culture media (Jurkowski and Cave, 1985; Karmali *et al.*, 1989; Rose *et al.*, 1995; Peterson *et al.*, 1998; Field *et al.*, 2000; Hardman, 2002; Robinson *et al.*, 2002). Linoleic acid (C18:2n-6, LA) and LNA (C18:3n-3) can be metabolized to arachidonic acid (C20:4n-6, AA) or EPA (C20:5n-3), respectively, by a system of elongase and desaturase enzymes. AA and EPA from membrane phospholipids can then be used as substrates for the biosynthesis of various eicosanoids, namely prostaglandins and thromboxanes via cyclooxygenase (COX) enzymes, and leukotrienes via lipoxygenase (LOX) enzymes (Figure 1-4).

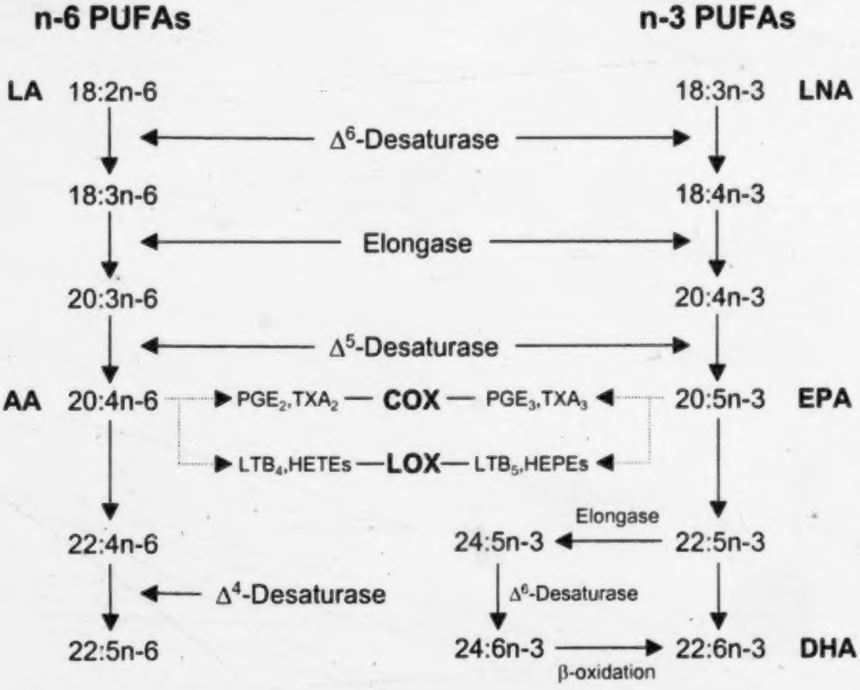

Figure 1-4 The metabolism of n-3 and n-6 polyunsaturated fatty acids. Each metabolic sequence competes for the same enzyme system. Abbreviations: LA, linoleic acid; LNA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; COX, cyclooxygenase; LOX, lipoxygenase. AA and EPA compete for conversion into prostaglandins, thromboxanes and leukotrienes by COX and LOX enzymes.

N-3 fatty acids may interfere with eicosanoid production through multiple mechanisms. N-3 fatty acids may decrease the supply of AA for conversion to eicosanoids by displacing AA from membrane phospholipids. Increasing the n-3 content of the diet or culture media has been shown to produce corresponding increases in the long chain n-3 PUFA content of cell membranes at the expense of n-6 PUFA, particularly AA (Rose, 1997), thus reducing the amount of AA in phospholipids available for eicosanoid production. N-3 PUFA also compete with n-6 PUFA for desaturases and elongases, and since n-3 PUFA have greater affinities for these enzymes than do n-6 PUFA, n-3 PUFA can reduce the elongation and desaturation of LA to AA (Larsson *et al.*, 2004). In addition, EPA may compete with AA for conversion to eicosanoids by the COX and LOX enzymes. Higher levels of n-3 PUFA in cell membranes reduce the production of proinflammatory eicosanoids (i.e. PGE_2, LTB_4, TXA_2) from n-6 PUFA, and increase the production of eicosanoids from n-3 PUFA (PGE_3, LTB_5). The eicosanoids produced from n-3 fatty acids have a lower biological potency than those produced from n-6 fatty acids (Hwang, 1989; Jump, 2002). There is also some evidence that n-3 fatty acids may actually decrease the expression or activity of the cyclooxygenase enzymes that are involved in eicosanoid production (Badawi *et al.*, 1998; Hamid *et al.*, 1999; Hardman, 2002).

Numerous studies have demonstrated that changing the dietary balance of n-3 and n-6 fatty acids can alter eicosanoid production by mammary tumors (Karmali *et al.*, 1984; Abou-El-Ela *et al.*, 1989; Rose *et al.*, 1995; Connolly *et al.*, 1997; Connolly *et al.*, 1999). This is not unexpected given the fact that n-3 and n-6 PUFA are metabolized by the same enzyme system, and can displace each other in cell membranes. However, it is not clear whether interfering with eicosanoid production has a detrimental effect on tumor growth, especially growth of mammary tumors (Rose and Connolly, 1999b). This is supported by studies that have shown significant decreases in eicosanoid (PGE_2) production with fish oil feeding without any detrimental effects on tumor growth (Fritsche and Johnston, 1990; Sasaki *et al.*, 1998), suggesting that tumor growth in these cases was not affected by decreased PGE_2 levels. In addition, studies involving chemical inhibitors of eicosanoid production are not entirely supportive. Cyclooxygenase inhibitors (eg. indomethacin, piroxicam, carprofen), administered to rats did not suppress mammary tumor growth despite inducing significant decreases in plasma and tumor PGE levels (Feldman and Hilf, 1985; Abou-El-Ela *et al.*, 1989; Carter *et al.*, 1989; Kitagawa and Noguchi, 1994). It is also unclear whether eicosanoids have direct effects on tumor cell proliferation *ex vivo*. Prostaglandins failed to stimulate mammary tumor cell growth *in vitro* (Balakrishnan *et al.*, 1989; Buckman *et al.*, 1991), and cyclooxygenase inhibitors have not consistently decreased mammary tumor growth *in vitro* (Fulton, 1984b; Buckman *et al.*, 1991), and even stimulated cell division in some cases, despite decreasing prostaglandin synthesis (Fulton, 1984a; Fulton, 1984b). It may be that the leukotriene class of eicosanoids are more important to the growth of mammary tumors than are the prostaglandins, as LOX inhibitors have been found more effective than COX inhibitors in inhibiting mammary tumor cell growth both *in vitro* (Rose and Connolly, 1990; Buckman *et al.*, 1991) and *in vivo* (Kitagawa and Noguchi, 1994).

1.3.2.2 N-3 PUFA may alter cell membrane-associated signal transduction

1.3.2.2.1 N-3 PUFA may alter physical properties of the cell membrane

N-3 fatty acids may alter physical properties of cell membranes, due to their long-chain, polyunsaturated structure. EPA and/or DHA were shown to increase the membrane fluidity of erythrocytes in healthy subjects consuming fish oil (Lund *et al.*, 1999) and of aortic endothelial cells in culture (Hashimoto *et al.*, 1999), and to increase the permeability of tumor cells *ex vivo* (Jenski *et al.*, 1991; Stillwell *et al.*, 1993). Such changes may be functionally relevant to membrane-associated signal transduction, perhaps by affecting the binding of important ligands (eg. cytokines, growth factors) to their receptors (Stubbs and Smith, 1984; Grimble and Tappia, 1995), or by affecting the function of signaling molecules in proximity to the membrane.

1.3.2.2.2 N-3 PUFA may alter the release of second messengers from the membrane

There is some evidence to suggest that n-3 fatty acids may alter the formation of phospholipid-derived second messengers such as diacylglycerol (DAG) and ceramide. DAG is derived from the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by phospholipase C (PLC) enzymes (Carpenter and Ji, 1999). DAG is the principle activator of several isoforms of protein kinase C (PKC), which is involved in a diverse array of cellular responses including proliferation (Hofmann, 2004). The sphingolipid ceramide is derived from the hydrolysis of sphingomyelin by sphingomyelinase (SMase) enzymes. Ceramide is believed to mediate anti-proliferative responses such as growth inhibition/cell-cycle arrest, apoptosis, differentiation, and senescence in response to certain cytokines or stress-causing agonists (Ogretmen and Hannun, 2004). Ceramide modulates components of various signaling pathways (eg. Akt, phospholipase D, PKC, and mitogen-activated protein kinases (MAPKs)) by regulating ceramide-activated protein phosphatases, kinases, and proteases (Ogretmen and Hannun, 2004). A small number of studies have reported a decrease in DAG and ceramide production by splenocytes from mice fed EPA and/or DHA (Fowler *et al.*, 1993; Jolly *et al.*, 1997; McMurray *et al.*, 2000). Conversely, ceramide levels were increased in Jurkat leukaemic cells exposed to DHA *in vitro* (Siddiqui *et al.*, 2003). These opposing findings may be related to differences in ceramide metabolism in normal versus transformed cells (levels of ceramide appear to be particularly lower in tumor cells; Ogretmen and Hannun, 2004), but clearly further research is needed to elucidate the effects of n-3 fatty acids on this interesting area of signal transduction.

1.3.2.2.3 N-3 PUFA may be incorporated into signaling molecules

All phospholipids and some of their second messengers such as DAG and ceramide contain fatty acyl chains. Therefore, it is possible that changing the fatty acid composition of these molecules may alter their function (Yaqoob, 1998). In support, it has been demonstrated that EPA and DHA are incorporated into signaling molecules such as DAG with the administration of n-3 PUFA in the diet (Fowler *et al.*, 1993; Marignani and Sebaldt, 1996), and there is some evidence that n-3 PUFA-enriched DAG is less potent in activating PKC than n-6 PUFA-enriched DAG (Bell and Sargent, 1987).

Many signaling molecules, including some tyrosine kinases, are reversibly acylated during signaling, which targets them to the cell membrane where they interact with other signaling molecules. It has been suggested that changing the fatty acid content of the diet (or the culture medium) may alter the acylation patterns of different signaling molecules (Sanderson and Calder, 1998; Hwang, 2000; Webb *et al.*, 2000), affecting their ability to interact with the membrane. Alternatively, it is possible that changes in membrane fatty acid composition induced by changes in diet could alter the physical nature of the membrane regions to which acylated signaling molecules bind (Sanderson and Calder, 1998), as described in the next section. Changes in early signal transduction events such as tyrosine kinase activation could be responsible for changes in other downstream signaling events that have been documented with n-3 PUFA administration.

1.3.2.2.4 N-3 PUFA may alter the lipid and protein composition of lipid rafts

A fairly recent finding in membrane biology is the proposal that biological membranes are composed of membrane microdomains that segregate together as lipid "rafts". Lipid rafts are operationally defined as cholesterol-dependent membrane microdomains resistant to solubilization by non-ionic detergents at low temperatures (Brown and London, 1998), and they have a number of pseudonyms in the literature (detergent-resistant membranes (DRMs), detergent-insoluble complexes (DICs), low density microdomains (LDMs), glycolipid-enriched membranes (GEMs), and Triton X-100-insoluble floating fractions (TIFFs) (Simons and Toomre, 2000). Lipid rafts are rich in sphingolipids and cholesterol, whereas phospholipids (compared to the plasma membrane) are relatively depleted and contain predominantly saturated fatty acid residues (Brown and Rose, 1992; Simons and Ikonen, 1997). Lipid rafts appear to be present in nearly all mammalian cell types (Brown and London, 1998). Because of their high lipid content, lipid rafts float to a low density during density gradient centrifugation (Simons and Ikonen, 1997). A subset of specialized rafts termed caveolae have also been described, which are flask-shaped structures in the membrane that are enriched in the protein caveolin-1, and are believed to have several functions including endocytosis, cholesterol transport and signal transduction (Ma *et al.*, 2004b).

Brown and Rose (1992) were the first to characterize the lipid composition of lipid rafts, isolated from canine kidney cells. They estimated about 10 % of the total cellular glycerophospholipids, versus 50 % of the total cellular sphingolipids were found in rafts. The molar ratio of cholesterol to glycerophospholipids to sphingolipids in rafts was reported by this group to be approximately 1:1:1. Phosphatidylethanolamine (PE), followed by phosphatidylcholine (PC), represented 19 and 11 % of the lipid material found in rafts, whereas phosphatidylserine (PS) and phosphatidylinositol (PI) made up 3 % and 2 % of raft material. Cholesterol represented ~32 % of the lipid material in rafts, whereas sphingomyelin (SM) represented ~14 % of the lipid material isolated from rafts.

Lipid rafts can include or exclude proteins to variable extents, leading to the hypothesis that rafts play a key role in signal transduction, perhaps functioning as platforms to concentrate signaling proteins (Simons and Toomre, 2000). Potentially, individual rafts may cluster together to connect interacting proteins into a signaling complex (Simons and Toomre, 2000). As well, rafts may protect signaling complexes from non-raft enzymes such as phosphatases that could otherwise affect the signaling process (Simons and Toomre, 2000).

A recent study by Foster *et al.* (2003) used a quantitative proteomics method to analyze raft protein composition. Their analysis revealed that many proteins isolated from lipid rafts had membrane associations, such as a transmembrane domain, or a hydrophobic modification such as a glycosylphosphatidylinositol (GPI) anchor, dual acylation, or palmitoylation. Their study confirmed the presence of molecules of tyrosine kinase cascade signaling pathways, as well as a significant number of serine/threonine kinases and phosphatases, and heterotrimeric G proteins. Overall, their study provides unbiased support for the involvement of lipid rafts as coordinators of signal transduction. The most extensive work relating to the function of rafts in signal transduction has come from the field of immunology, where there is strong evidence that lipid raft integrity is required for optimal T cell receptor signal transduction and immune response (Simons and Toomre, 2000). Signaling through B cell, EGF, insulin, and integrin receptors is also thought to involve rafts (Simons and Toomre, 2000).

The size of individual rafts is largely unknown but hotly debated. Foster *et al.* (2003) suggest from their analysis that the percentage of the membrane occupied by rafts is considerably less than 10 % (based on the enrichment of raft proteins as a fraction of the total membrane protein). However, it should be noted that this does not imply that rafts are of a uniform or static size and composition. While individual rafts are thought to be quite small, it has been suggested that they may fuse to form larger domains (eg. upon receptor-ligand binding) (Cherukuri *et al.*, 2001). It should also be noted that the detergent-resistant method of isolating lipid rafts is not able to isolate individual rafts or ligand-activated raft clusters in their native state (Simons and Toomre, 2000).

Relatively little is known about the effects of PUFA on lipid rafts, especially in tumor cells. Stulnig *et al.* demonstrated that PUFA treatment (both LA and EPA) of Jurkat T cells *in vitro* led to a displacement of the Src family kinases Lck and Fyn from lipid rafts, compared to treatment with saturated or monounsaturated fatty acids (Stulnig *et al.*, 1998). Subsequently, Stulnig *et al.* showed that EPA was significantly incorporated into lipid rafts (albeit to a lesser extent than into whole membrane phospholipids) following EPA treatment of Jurkat T cells *in vitro* (Stulnig *et al.*, 2001). This was associated with a displacement of LAT (linker for activation of T cells) from T cell rafts (Stulnig *et al.*, 2001), and a subsequent decrease in LAT-regulated phosphorylation of PLCγ (Zeyda *et al.*, 2002). Similarly, Diaz *et al.* (2002) reported that DHA treatment of human peripheral blood mononuclear cells *in vitro* resulted in a displacement of phospholipase D (PLD) from lipid rafts, concomitant with an increase in PLD activity. The latter two studies are significant as they demonstrate that exclusion of certain signaling proteins from lipid rafts affects their function. Such changes in protein activity, as a result of either raft inclusion or exclusion, may be caused by conformational changes in these proteins when they are exposed to a different membrane environment, or they may be caused by interactions with other proteins within or outside of rafts (Diaz *et al.*, 2002).

Two studies have examined PUFA effects on rafts in an *in vivo* situation. Fan *et al.* (2003) demonstrated that EPA and DHA were selectively incorporated into the inner cytoplasmic leaflet phospholipids (i.e. PS and PE), and decreased sphingomyelin content, of rafts from T cells of fish oil-fed mice. This was the first study to demonstrate an effect of dietary n-3 PUFA on raft lipid composition, and in particular, on the distribution of individual phospholipid classes in rafts. Dietary EPA and DHA have since been shown to be incorporated into caveolae from colonocytes of fish oil-fed mice (Ma *et al.*, 2004a). Enrichment of n-3 PUFA into caveolae was associated with a reduction in caveolae cholesterol, as well as a displacement of H-ras from caveolae, accompanied by a suppression of H-ras activity.

It is not clear how PUFA regulate raft localization of proteins, but it has been proposed that the substitution of unsaturated fatty acid residues for saturated resides in rafts may interfere with the interaction of acylated (eg. palmitoylated, myristoylated) proteins with nonpolar lipids (Stulnig *et al.*, 2001). Alternatively, PUFA may alter the acylation of proteins, impacting on the ability of the protein to localize within rafts. This idea is supported by a study by Webb *et al.* (2000) who showed that both AA and EPA dramatically decreased Fyn acylation by palmitate, and subsequent raft localization, in COS-1 cells exposed to AA and EPA *in vitro*. The authors speculated that palmitate may have been replaced by AA and EPA. Liang *et al.* (2001) demonstrated that acylation of the Src family kinase Fyn with oleic acid (18:1) decreased its localization in rafts of COS-1 cells, suggesting that acylation of proteins by unsaturated fatty acids, instead of saturated fatty acyl moieties, may affect raft localization. However, in this study, the oleic acid-labeled form of Fyn represented only a very small proportion of the total Fyn population (Liang *et al.*, 2001), suggesting that this may not be the predominant mechanism by which unsaturated fatty acids alter raft protein composition. In support, Stulnig *et al.* (2001) demonstrated that both the palmitate-labeled and unlabeled forms of Lck and LAT were displaced from rafts of PUFA-treated T cells to a similar extent, arguing that alterations in the lipid raft environment are more important than PUFA-acylation of proteins in raft protein localization.

To date, no one has examined the effects of PUFA on lipid rafts in tumor cells. However, the evidence presented above suggests that n-3 PUFA modification of raft lipid environment, leading to alterations in signal transduction, is a plausible mechanism to describe the anticancer effects of n-3 PUFA.

1.3.2.3 N-3 PUFA may modulate gene expression

There is considerable evidence to indicate that n-3 PUFA are capable of inducing changes in gene expression in a number of different cell types, including tumor cells. The list of genes whose expression appears to be affected by fish oil or purified n-3 fatty acids continues to grow and excellent reviews have been published (Jump *et al.*, 1996; Price *et al.*, 2000; Jump, 2002; Clarke *et al.*, 2002). As examples, n-3 PUFA treatment has been associated with: reduced expression of prointerleukin-1β gene transcription by

LPS-stimulated spleen cells from fish oil-fed mice (Urakazi *et al.*, 1993); reduced transcription of the c-fos gene in cultured smooth muscle cells by EPA (Terano *et al.*, 1996); suppressed IL-2 receptor alpha mRNA levels in splenic lymphocytes of mice fed EPA and DHA (Jolly *et al.*, 1998); reduced PGH synthase expression and levels of PGH synthase mRNA levels by EPA- and DHA-treated bovine aortic endothelial cells (Achard *et al.*, 1997); suppressed type IV collagenase mRNA expression in EPA-treated MDA-MB-435 breast cancer cells (Liu and Rose, 1995); reduced hepatic S14 mRNA levels in rats fed fish oil (Jump *et al.*, 1993); reduced p21^{ras} and COX-2 mRNA expression in mammary glands of rats fed fish oil (Badawi *et al.*, 1998).

The exact way in which n-3 fatty acids alter gene transcription is not known, but there is considerable speculation and new evidence that this might involve a class of nuclear receptors called peroxisome proliferator-activated receptors (PPARs). PPARs are ligand-activated transcription factors present in a variety of cell types, with diverse actions, mainly in lipid metabolism (Kliewer and Willson, 1998; Yaqoob, 2003). The PPAR family has at least three closely related members – PPARα (expressed in liver, kidney, heart and muscle), PPARγ (expressed in fat cells, large intestine, monocytic cells, and recently identified in a number of breast cancer cell lines), and PPARβ/δ (expressed in nearly all tissues) (Kliewer *et al.*, 1999). The peroxisome proliferator-activated receptors are fatty acid and eicosanoid receptors (Kliewer *et al.*, 1999). Various fatty acids have been shown to bind and activate the PPARs (Krey *et al.*, 1997; Sarraf *et al.*, 1998; Murakami *et al.*, 1999), and they can also be activated by certain cyclooxygenase and lipoxygenase metabolites of polyunsaturated fatty acids (Kliewer *et al.*, 1999). Recently, it was demonstrated that long-chain PUFA, including EPA, can bind to and activate PPARs (Murakami *et al.*, 1999; Xu *et al.*, 1999), providing a mechanism by which n-3 PUFA could directly regulate gene expression.

PPARγ is known for its role in inducing differentiation of adipocytes, but it appears that PPARγ may also play a role in inhibiting carcinogenesis. Ligand activation of PPARγ in colon tumor cell lines decreases tumor cell growth and induces differentiation (reviewed by Sarraf *et al.*, 1998). Similar effects have also been observed in breast cancer cells (Mueller *et al.*, 1998; Elstner *et al.*, 1998), and in one manuscript, activation of PPARγ also induced apoptosis (Clay *et al.*, 1999). Sarraf *et al.* identified loss-of-function mutations in the PPARγ gene in a number of human colon cancers, suggesting that PPARγ may function as a tumor suppressor (Sarraf *et al.*, 1999). However, activation of PPARγ has been found to promote the development of colon tumors in a mouse model (see Gelman *et al.*, 1999). In addition, another study found that lower transcriptional activity of PPARγ led to increased proliferation, whereas higher transcriptional activity of PPARγ led to cell cycle arrest and apoptosis of human breast cancer cells (Clay *et al.*, 2001). Thus, further research is clearly needed to elucidate the role of PPARγ in tumor development.

N-3 PUFA also have been shown to modulate the activity of the transcription factor NFκB in monocytes and macrophages (Weber *et al.*, 1995; Lee *et al.*, 2003; Novak *et al.*, 2003; Zhao *et al.*, 2004; Lee *et al.*, 2004). NFκB is a ubiquitously expressed transcription factor that regulates the expression of a number of different genes relating to cell survival and programmed cell death (Nicholson and Anderson, 2002). It is generally accepted that NFκB functions to promote cell survival and oppose apoptosis. The active transcription factor generally consists of a homo- or heterodimer of NFκB family member subunits (eg. RelA (p65), p50, p105, p100). In resting cells, NFκB is sequestered in the cytoplasm through association with its inhibitor, IκB (inhibitor of kappa B). Upon cell stimulation with cytokines (eg. IL-1, TNF), growth factors (eg. EGF, PDGF, insulin), or various stressors (eg. UV radiation, pH, hypoxia, reactive oxygen species), the inhibitor of IκB kinase (IKK) is activated by upstream kinases such as Akt (also known as protein kinase B, or PKB), which phosphorylates IκB, targeting it for ubiquitination and degradation by the ubiquitin proteasomal system. This leads to the dissociation of NFκB from its inhibitor, allowing NFκB to translocate to the nucleus where it can transcribe genes (Nakshatri and Goulet Jr., 2002). Genes induced upon NFκB activation include cyclin D1, cyclin B1, COX-2, bcl-2, Akt1, cIAP2. It is not clear how n-3 PUFA modulate NFκB activity, however, it has been suggested that these fatty acids modulate the activity of upstream signaling molecules involved in the activation of NFκB (Weber *et al.*, 1995; Lee *et al.*, 2003; Novak *et al.*, 2003; Zhao *et al.*, 2004).

1.3.2.4 N-3 PUFA may increase the formation of cytotoxic lipid peroxidation products

Polyunsaturated fatty acids with two or more double bonds are highly susceptible to free radical attack, ultimately leading to the formation of lipid hydroperoxides. Lipid peroxidation is initiated by hydrogen abstraction from an unsaturated fatty acid by reactive oxygen species (ROS). The resulting lipid radical reacts with oxygen to form a fatty acid peroxyl radical, which can attack adjacent fatty acid chains in the cell membrane, and thus propagate lipid peroxidation (Halliwell, 1994). The major effects of the products of lipid peroxidation are inhibition of DNA synthesis, cell division and tumor growth, and induction of tumor cell death (Galeotti *et al.*, 1986; Girotti, 1998).

The long-chain n-3 PUFA are particularly susceptible to lipid peroxidation, leading to the hypothesis that their tumor growth-inhibitory effects may occur through the production of lipid peroxidation products. The most convincing evidence for this mode of action comes from *in vitro* and *in vivo* studies demonstrating that the addition of antioxidants such as vitamin E (which functions to prevent lipid peroxidation; De Vries and Van Noorden, 1992) to either the cell culture medium or the diet of animals abrogates the anti-cancer effect of n-3 fatty acids, whereas the addition of prooxidants can augment the anti-cancer effect of n-3 fatty acids (Begin *et al.*, 1985; Gonzalez *et al.*, 1991; Gonzalez *et al.*, 1993; Chajes *et al.*, 1995; Cognault *et al.*, 2000). However, not all studies support lipid peroxidation as the mechanism for n-3 PUFA-induced growth inhibition, as no increase in lipid peroxidation was observed with n-3 PUFA treatment of MCF-7 mammary tumor cells *in vitro* in one study (Grammatikos *et al.*, 1994), whereas the addition of vitamin E (a lipid antioxidant) did not abrogate the effects of n-3 PUFA in culture in another (Chamras *et al.*, 2002). Clearly more research is needed to clarify whether lipid peroxidation products play a role in the growth-inhibitory effects of n-3 PUFA.

The mechanism by which lipid peroxidation products could inhibit tumor growth is not clear, but may involve damage to cell membranes, inactivation of membrane transport systems or membrane bound enzymes, or damage to other cellular proteins and DNA (Welsch, 1995). Lipid peroxidation is reported to enhance the expression of the Fas death receptor and suppress bcl-2 expression, perhaps explaining some reports of peroxidation-induced apoptotic cell death (Das, 1999). Interestingly, there appears to be a relationship between lipid peroxidation and cell growth rate. Highly undifferentiated tumor cells have a very low degree of lipid peroxidation which seems to be inversely related to their growth rate, such that when DNA synthesis is at a maximum, lipid peroxidation is suppressed, and vice versa (De Vries and Van Noorden, 1992; Gonzalez, 1995). Furthermore, rapidly growing normal tissues (eg. testis, bone marrow and intestinal epithelium) appear resistant to peroxidation, with low levels of peroxidation products (Masotti *et al.*, 1988; Gonzalez, 1995). These observations suggest that lipid peroxidation products may be involved in the control of cell division (Gonzalez, 1995).

1.4 SUMMARY

Animal and cell culture work has resulted in convincing evidence that the long-chain n-3 PUFA can inhibit the growth of mammary tumors. However, it is not clear how n-3 fatty acids influence tumor growth, even at a basic level (i.e. whether n-3 PUFA affect proliferation and/or tumor cell death). Many mechanisms have been proposed to explain the growth-inhibitory effects of n-3 PUFA, but this field is still not well understood. Understanding the mechanism(s) by which n-3 PUFA inhibit tumor growth is important for determining their clinical importance in cancer, especially when incorporated with other therapies.

There is a growing interest in the use of dietary intervention in the treatment of breast cancer both from patients and health professionals. Treatment with dietary fats is an inexpensive and non-invasive method of treatment. There are currently no evidence-based recommendations for specific dietary lipids (i.e. n-3 PUFA) for individuals with, or at high risk of, breast cancer, as there are for other chronic diseases such as cardiovascular disease. N-3 fatty acids are generally regarded as safe compounds, and their growth-inhibitory effects appear to be limited to cancerous cells. A number of groups have tested the effects of n-3 PUFA on non-cancerous cells *in vitro* (human fibroblasts, human mammary epithelial cells, canine kidney cells), and report that n-3 PUFA (provided at concentrations that inhibit the growth of tumor cells) exert little or no cytotoxic effects on non-tumorigenic cells (Begin *et al.*, 1985; Begin *et al.*, 1986; Grammatikos *et al.*, 1994; Chajes *et al.*, 1995; Bernard-Gallon *et al.*, 2002). In humans, n-3 PUFA are well tolerated even at high doses (i.e. up to 0.3 g EPA/kg body weight per day in a phase I clinical trial), with relatively mild side effects including bloating and a fishy taste in the mouth (Burns *et al.*, 1999). Thus n-3 fatty acids, in combination with standard treatments, may offer a nontoxic means to improve cancer treatment outcomes and potentially even slow or prevent cancer recurrence.

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2. STUDY RATIONALE

2.1 RATIONALE

It is well established that dietary n-3 fatty acids decrease mammary tumor growth promotion in a number of carcinogen-induced, transplantable, spontaneous and metastatic animal tumor models as well as breast cancer cell lines (see Tables 1-1 and 1-2). This is supported by epidemiological work in human populations. However, despite the large number of studies conducted on the growth-inhibitory effects of n-3 PUFA on mammary tumors, there is little consensus among the published reports as to the mechanism(s) by which n-3 PUFA inhibit mammary tumor growth (see Tables 1-1 and 1-2). Few of the published reports have even attempted to determine whether n-3 PUFA affect primarily tumor cell proliferation or tumor cell death, or both outcomes. This initial step is important for focusing subsequent studies on the mechanisms of n-3 PUFA-induced tumor growth inhibition.

Many mechanisms have been proposed to explain the growth-inhibitory effects of n-3 PUFA on tumors, including changes in eicosanoid production, alteration of cell membrane-associated signal transduction, modulation of gene expression, and formation of lipid peroxidation products. Modulation of eicosanoid production by n-3 PUFA has been very well studied. Nevertheless, it is possible that decreases in arachidonic acid-derived eicosanoids observed with n-3 PUFA administration is a consequence of n-3 PUFA enrichment in cell membranes, and may have indirect effects on tumor growth rather than directly influencing proliferation or apoptosis of mammary tumors. It is quite likely that n-3 PUFA incorporation into cell membranes alters membrane-associated signal transduction (eg. events leading to cell growth or death), but still relatively little is known about this mechanism. Along this line, very little is known about the effects of n-3 PUFA on membrane raft microdomains, which have been recently implicated as signaling hot spots. It is quite possible that modulation of the activity of upstream signaling molecules by n-3 PUFA are partially responsible for changes in gene expression observed with n-3 PUFA administration. Finally, effects of n-3 PUFA via lipid peroxidation are still a hypothesis and remain to be further elucidated. In general, it is well accepted that n-3 PUFA are incorporated into cell membranes where they can potentially affect a number of membrane properties. This likely represents a common fundamental mechanism by which n-3 PUFA exert their effects on a number of different cell types and organ systems.

The overall objective of this research is to determine the cellular and molecular mechanisms by which the long chain n-3 PUFA, EPA and DHA, inhibit the growth of established human breast cancer cell lines. It is important to understand the mechanism by which n-3 PUFA inhibit mammary tumor growth as this lends credibility to their use as anti-cancer agents, and is critical for determining how these fatty acids may interact with current anti-cancer therapies. There is promising data (mostly from animal and cell culture work) to indicate that n-3 PUFA enhance the cytotoxicity of some anti-neoplastic agents, and may also limit the toxic side effects of chemotherapy (reviewed by Baracos *et al.*, 2004). Thus there is great interest in, and potential for, the use of n-3 fatty acid supplementation as an effective adjuvant for current chemotherapy protocols. The results of the current research will be important for focusing animal and human trials involving the use of dietary n-3 PUFA as adjuvants for cancer treatment, and may identify new drug targets for combination with n-3 PUFA treatment.

2.2 SELECTION OF TUMOR MODEL

Human breast cancer cell lines were chosen as the model to investigate mechanisms of fatty acid-induced tumor growth inhibition as they enable us to focus on the direct impact of n-3 PUFA on tumor cell growth and death. Information obtained in an *in vitro* system will generate the hypotheses and provide the parameters to study in future animal and clinical studies. The number of available breast cancer cell lines is relatively small, but it was recently concluded in a comprehensive review that breast cancer cell lines are likely to reflect, to a large extent, the features of cancer cells *in vivo* (Lacroix and Leclercq, 2004). In the research presented in this thesis, the effects of n-3 PUFA were examined on two phenotypically dissimilar human breast cancer cell lines, MDA-MB-231 and MCF-7, to confirm that the growth-inhibitory effects of n-3 PUFA are not limited to one particular cell line but may be relevant to a broader spectrum of mammary tumors.

The MDA-MB-231 and MCF-7 cell lines are two of the most widely studied and best characterized human breast cancer cell lines (Lacroix and Leclercq, 2004). Characteristics of these cell lines are presented in Table 2-1. The MCF-7 cell line was derived from a patient with metastatic breast cancer in 1970 (Soule *et al.*, 1973), and has been studied longer than any other breast cancer cell model system (Simstein *et al.*, 2003). MCF-7 cells are estrogen receptor (ER)-positive, and this cell line has become the prototype for ER-positive breast cancer (Simstein *et al.*, 2003). MCF-7 cells require estrogen for tumorigenesis *in vivo* (Dickson *et al.*, 1986), and are poorly metastatic (Timoshenko *et al.*, 2003). The MDA-MB-231 cell line was derived from a patient with metastatic breast cancer in 1973 (Cailleau *et al.*, 1974). Unlike MCF-7 cells, MDA-MB-231 cells are ER-negative, do not require estrogen for tumorigenesis *in vivo*, and are highly invasive (Dickson *et al.*, 1986; Timoshenko *et al.*, 2003). When transplanted into nude mice, MDA-MB-231 cells are very aggressive and result in metastases in the lungs and lymph nodes (Lacroix and Leclercq, 2004). This cell line is believed to be representative of ER-negative tumors, which comprise about 30 % of human breast tumors.

Preliminary experiments were conducted in both cell lines to determine optimal growth-inhibitory concentrations of EPA and DHA in the absence and presence of LA (see Appendix). A mixture of EPA and DHA, the two primary n-3 fatty acids found in fish oils, were provided in a 3:2 ratio (EPA:DHA), as this approximates the average ratio of EPA to DHA found in most commercially available fish oils used in the animal studies of dietary n-3 PUFA and mammary tumor growth (see Table 1-1). The majority of studies conducted *in vitro* are not able to demonstrate tumor growth inhibition by n-3 PUFA in the presence of the essential fatty acid, linoleic acid, which would be present *in vivo* and is required for mammary tumorigenesis in the rat (Ip *et al.*, 1985). Therefore, all experiments conducted in this research consisted of four fatty acid treatments (based on the preliminary experiments described in the Appendix): LA (75 μM), found to promote optimal cell growth; EPA and/or DHA (100 μM total), found to optimally inhibit the growth of cells without cytotoxic effects; EPA and/or DHA plus LA (150 μM total), to

Table 2-1 Characteristics of the MCF-7 and MDA-MB-231 human breast cancer cell lines[1]

Feature	MCF-7	MDA-MB-231	Reference
Type of cancer	invasive ductal carcinoma	invasive ductal carcinoma	Lacroix and Leclercq, 2004
Original tissue source	metastasis (pleural effusion)	metastasis (pleural effusion)	Lacroix and Leclercq, 2004
Phenotype	"luminal epithelial-like"	"mesenchymal-like" or "stromal-like"; fibroblastoid	Lacroix and Leclercq, 2004
Primary tumor type	N/A	grade III, poorly-differentiated	Lacroix and Leclercq, 2004
Modal chromosome number	65, 79, 86, 88	64, 69-70	Lacroix and Leclercq, 2004
Tumorigenicity *in vivo*	requires estrogen		Dickson *et al*., 1986
Invasiveness *in vitro*	weak	high	Lacroix and Leclercq, 2004
Metastatic potential	poorly metastatic	highly metastatic	Timoshenko *et al*., 2003; Lacroix and Leclercq, 2004
Approx. cell doubling time	36 hours	16-20 hours	Engel and Young, 1978; Schneider *et al*., 1990
ER status	+	-	Lacroix and Leclercq, 2004
PgR status	+	-	Lacroix and Leclercq, 2004
EGFR expression	low	high	Anderson *et al*., 2001; Lacroix and Leclercq, 2004
erb-b2/Her-2/neu expression	low	low	Anderson *et al*., 2001
IGF-IR expression	high	low	Bartucci *et al*., 2001
Akt-3 expression	low	high	Lacroix and Leclercq, 2004
basal NFκB activity	low	high, constitutive	Sliva *et al*., 2002
p53 expression[2]	+/+	mut/-	Runnebaum *et al*., 1994; Elstner *et al*., 1995; Xu and Loo, 2001
Other features:	This cell line does not express caspase-3, is caveolin-1 deficient, and lacks $^{\Delta}$4-, $^{\Delta}$5-, and $^{\Delta}$6-desaturase activity	This cell line has constitutively active PI3K, an activating K-ras mutation, and high constitutive COX-2 expression	Kurokawa *et al*., 1999; Liang *et al*., 2001; Grammatikos *et al*., 1994; Lee *et al*., 1998; Gilhooly and Rose, 1999; Liu and Rose, 1996; Sliva *et al*., 2002

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[1] Abbreviations: COX, cyclooxygenase; ER, estrogen receptor; EGFR, epidermal growth factor receptor; IGF-IR, insulin-like growth factor I receptor; N/A, not available; PgR, progesterone receptor; PI3K, phosphatidylinositol 3-kinase

[2] The p53 gene in MDA-MB-231 cells is deleted in one allele and mutated in the other, resulting in non-functional p53 protein. MCF-7 cells express wild-type p53.

demonstrate growth-inhibitory effects of EPA and DHA in the presence of LA; and LA (150 μM), a fatty acid concentration control to ensure that the total fatty acid concentration was not cytotoxic to the cells.

Based on fatty acid composition analysis of our fetal calf serum (FCS), it was not expected that serum fatty acids would interfere with the effects of the exogenous fatty acids used in the present research. Our analysis revealed that media prepared with 5 % v/v FCS contained approximately 3 μM LA, and less than 1 μM of either EPA or DHA (Ma *et al.*, 2002). These fatty acid concentrations found in serum are negligible compared to the 75 to 150 μM of exogenous fatty acids applied in the present research, and are also constant across all treatments, so they are unlikely to affect tumor growth regulation induced by the experimental fatty acids.

The concentrations of EPA and DHA used in the present study (75 to 100 μM) are well within the range of plasma concentrations of EPA and DHA reported in humans consuming fish or fish oil supplements. In healthy subjects, the fasting plasma concentrations of EPA and DHA following 4 weeks of oral administration of a fish oil supplement (1.4 g EPA + 0.6 g DHA/day) were 209 μg/ml EPA (~ 690 μM) and 115 μg/ml DHA (~ 350 μM) (Hirai *et al.*, 1982). Among breast cancer patients consuming 10 g of fish oil per day for 3 months, fasting plasma concentrations of EPA and DHA were 686 μM and 529 μM, respectively (Bagga *et al.*, 1997). Postmenopausal Japanese women not consuming any supplements had plasma EPA and DHA levels of approximately 70 μg/ml EPA (~ 230 μM) and 130 μg/ml DHA (~ 400 μM), likely a reflection of higher dietary n-3 fatty acid intake in this population (Sumino *et al.*, 2003). It should be noted, however, that it is not known how plasma concentrations of EPA and DHA translate into actual tissue concentrations of EPA and DHA available to breast cells, therefore comparisons to plasma levels of EPA and DHA can only be made at this time.

2.3 OBJECTIVES AND HYPOTHESES

The objectives of this research are to test the following general working hypotheses:

I. Long-chain n-3 fatty acids inhibit mammary tumor growth *in vitro* through a reduction in cellular proliferation, an increase in apoptosis, or a combination of both.

II. Long-chain n-3 fatty acids are incorporated into mammary tumor cell membrane phospholipids and alter the function of cell survival signaling pathways.

III. Long-chain n-3 fatty acids are incorporated into mammary tumor cell membrane lipid rafts, and alter raft lipid and signaling protein composition, leading to functionally relevant changes in signal transduction.

2.4 CHAPTER FORMAT

The hypotheses posed were tested in a sequence of experiments. These experiments are organized as thesis chapters and have been prepared for submission for scientific publication as individual papers.

Chapter 3 examines the effects of EPA and DHA on tumor cell proliferation and death, and on signaling through the Akt/NFκB cell survival signaling pathway, in both the MDA-MB-231 and MCF-7 cell lines (*hypotheses 1 and 2*). It was hypothesized that: a combination of EPA and DHA will reduce cellular proliferation ([methyl-^{3}H]-thymidine incorporation, expression of proliferation-related and cell cycle regulatory proteins) relative to the control; EPA and DHA will induce apoptotic cell death; EPA and DHA will be incorporated into tumor cell membrane phospholipids; EPA and DHA will decrease Akt phosphorylation; EPA and DHA will reduce NFκB activity; the effects of EPA and DHA are not due to a deficiency of LA in the cell culture media and are still achieved when EPA and DHA are provided in combination with LA; and the effects of EPA and DHA are not related to increased lipid peroxidation.

Chapter 4 describes the development of a microscale method for the isolation of lipid rafts from both MDA-MB-231 and MCF-7 cells. At the time of conducting this research, there were no micromethods available to isolate lipid rafts from tumor cells. The development of this micro method was required to test hypothesis 3.

Chapter 5 describes the effects of EPA and DHA on membrane raft fatty acid composition and mass distribution of phospholipids in rafts from both MDA-MB-231 and MCF-7 cells, as well as raft signaling protein composition in MDA-MB-231 cells (*hypothesis 3*). It was hypothesized that: EPA and DHA will be incorporated into membrane lipid rafts of both cell lines; EPA and DHA will alter the mass distribution of phospholipids within lipid rafts of both cell lines; and EPA and DHA will displace signaling proteins involved in tumor growth (epidermal growth factor receptor, Akt, phosphatidylinositol 3-kinase, phospholipase Cγ) from lipid rafts, which will decrease their activity.

Chapter 6 summarizes the findings of these hypotheses and provides general discussion.

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3. INHIBITION OF MDA-MB-231 AND MCF-7 BREAST CANCER CELL GROWTH BY EICOSAPENTAENOIC ACID AND DOCOSAHEXAENOIC ACID[1,2]

3.1 INTRODUCTION

The role of dietary fat in breast cancer development and/or promotion is not well understood, and remains controversial. While there is conflicting evidence regarding total dietary fat intake and breast cancer risk, ecological studies have found an inverse relationship between breast cancer risk and fish or fish polyunsaturated fatty acid (PUFA) consumption (see de Deckere, 1999 for review), suggesting that fish fatty acids may have a protective effect against breast cancer development.

Fish oils contain large amounts of both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), long-chain omega-3 (or n-3) PUFA. The inhibitory effects of EPA and DHA on tumor promotion have been well documented in rodent models of breast cancer. When provided in the diet through fish oils, these fatty acids decrease the incidence as well as the growth and metastasis of chemically-induced and transplantable mammary tumors (see Rose and Connolly, 1999 for review). The relative potency of EPA versus DHA in inhibiting tumor growth is not clear but is important to establish as these fatty acids are now available commercially as supplements and in enriched foods.

There are fewer studies published that describe the effects of EPA and DHA *in vitro*, but the available data consistently show that these two fatty acids inhibit the growth of several different breast cancer cell lines (Begin *et al.*, 1986; Begin *et al.*, 1988; Rose and Connolly, 1990; Grammatikos *et al.*, 1994b; Chajes *et al.*, 1995; Noguchi *et al.*, 1995; Abdi-Dezfuli *et al.*, 1997; Senzaki *et al.*, 1998; Yamamoto *et al.*, 1999; Chamras *et al.*, 2002). However, most of the *in vitro* studies have not included the essential n-6 PUFA linoleic acid (LA) in the growth medium, a fatty acid that would be present *in vivo*

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[1] A version of this chapter has been accepted for publication in *Breast Cancer Research and Treatment*.

[2] This work was presented in part at Experimental Biology 2003, San Diego, CA, April 2003, and published in part in abstract form. (Schley, P.D., Robinson, L.E., & Field, C.J. Eicosapentaenoic acid and docosahexaenoic acid induce apoptosis in MDA-MB-231 and MCF-7 human breast cancer cells. FASEB J. 17: A1373.)

and is required for mammary tumorigenesis in the rat (Ip *et al.*, 1985). These studies are not able to confirm that tumor growth inhibition by n-3 PUFA occurs in the presence of abundant LA, which is an important consideration addressed in this research.

Despite the abundance of evidence both *in vivo* and *in vitro* that n-3 fatty acids inhibit mammary tumor growth promotion, the mechanism(s) by which n-3 PUFA exert their anticancer effect is not well understood. Decreased growth of cancerous cells can occur either by the inhibition or reduced rate of cellular proliferation, and/or by the induction of necrotic or apoptotic cell death. While many studies have shown decreased tumor promotion with exposure to EPA and/or DHA, few studies have attempted to determine whether cell proliferation or cell death, or both, are affected by n-3 PUFA.

It has been established that n-3 PUFA are incorporated into tumor cell membrane phospholipids when provided in the diet (see Jurkowski and Cave, 1985; Karmali *et al.*, 1989; Rose *et al.*, 1995; Robinson *et al.*, 2002), but the biological impact of this has not been established. While a number of studies have examined n-3 PUFA incorporation into total membrane lipids, less is known about n-3 PUFA incorporation into individual membrane phospholipids, which are important in membrane signaling and receptor-mediated functions.

In addition, little is known about the effects of n-3 PUFA on the signal transduction pathways leading to cell proliferation and death. One such pathway is the Akt cell survival signaling pathway, the disruption of which is a frequent occurrence in many human cancers including breast cancer (Nicholson and Anderson, 2002). Akt, also known as protein kinase B (PKB), is a serine/threonine kinase that is activated in response to cytokines and growth factors (Vivanco and Sawyers, 2002). Akt activation occurs through its translocation to the plasma membrane and phosphorylation at two key residues. Phosphorylation of Thr308 is necessary and sufficient for Akt activation, but maximal activation requires the additional phosphorylation of Ser473 (Vivanco and Sawyers, 2002). Akt is believed to be an important regulator of cell proliferation and survival (Vivanco and Sawyers, 2002). Akt functions directly to promote cell survival and protect cells from apoptotic cell death by phosphorylating and inactivating components of the cell death machinery (eg. caspase-9, BAD). In addition, Akt can indirectly promote cell survival by activating prosurvival transcription factors such as

NFκB. NFκB also plays a role in protecting cells from apoptosis and promoting cell growth by inducing the transcription of prosurvival and anti-apoptotic genes (eg. cyclin D1, cyclin B1, bcl-2, PCNA) (Nicholson and Anderson, 2002; Wang *et al.*, 2002). Interestingly, in breast cancer cells there appears to be a correlation between increased NFκB activity in tumors and increased aggressiveness or invasiveness (Nakshatri *et al.*, 1997). NFκB has been found to be constitutively active in many human breast tumors and cell lines (particularly estrogen receptor-negative, hormone-independent cells) including the MDA-MB-231 cell line (Nakshatri *et al.*, 1997), and may contribute to tumorigenesis.

In this study, we examined the effects of EPA and DHA on the growth of two human breast cancer cell lines, MDA-MB-231 and MCF-7, to determine the mechanism by which these fatty acids inhibit breast cancer cell growth. We employed a number of assays estimating the effect of EPA and DHA on cell proliferation ([methyl-^{3}H]-thymidine uptake, expression of proliferation-associated proteins, cyclin protein expression) versus cell death (detection of activated caspases, loss of mitochondrial membrane potential, detection of DNA fragmentation, lactate dehydrogenase release, propidium iodide uptake) in an attempt to identify the mechanism for n-3 PUFA-induced tumor cell growth inhibition. We also analyzed the fatty acid composition of individual membrane phospholipids in both cell lines following incubation with n-3 PUFA. In addition, we examined the effects of n-3 PUFA on the Akt cell survival signaling pathway, by measuring the phosphorylation state of the Akt kinase, as well as the DNA-binding activity of NFκB. We conclude that the long-chain n-3 PUFA impair proliferation and induce apoptotic cell death in MDA-MB-231 and MCF-7 cells. EPA and DHA are significantly incorporated into membrane phospholipids in both cell lines. Decreased tumor cell survival may result from impaired Akt phosphorylation and NFκB activity, perhaps due to n-3 PUFA-induced alterations in cell membrane properties.

3.2 MATERIALS AND METHODS

Cell culture

The MDA-MB-231 and MCF-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in 75 cm^2 tissue culture flasks (Fisher

Scientific, Edmonton, Alberta, Canada). All media and media components were obtained from Gibco Invitrogen Corporation (Burlington, Ontario, Canada) unless otherwise stated. MDA-MB-231 cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 1 % v/v antibiotic/antimycotic solution (1 x 10^5 U/L penicillin, 100 mg/L streptomycin, and 25 mg/L amphotericin B) and 5 % v/v fetal calf serum (FCS). MCF-7 cells were grown in minimum essential medium (MEM) supplemented with 1 % v/v antibiotic/antimycotic solution, 5 % v/v FCS, 1 mM sodium pyruvate, and 10 µg/mL bovine insulin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Cells were grown at 37°C in 5 % CO_2 at 98 % relative humidity. The culture media was changed every two days, and cells were passaged into new flasks with fresh media when monolayers reached approximate confluency (determined by viewing monolayers under an inverted microscope).

Fatty acids

EPA (C20:5n-3) was purchased from NuChek Prep Inc. (Elysian, MN). DHA (C22:6n-3) was purchased from Martek Biosciences Corp. (Columbia, MD). Linoleic acid (LA, C18:2n-6) was prepared from safflower oil in our laboratory as described previously (Ma *et al.*, 1999). Fatty acids were dissolved in ethanol and stored at –80 °C under N_2(g).

Preparation of fatty acid-supplemented media

Appropriate volumes of LA, EPA or DHA stock solutions (10 mg/mL in ethanol) were dried under N_2(g) in 15-mL conical tubes (Fisher Scientific, Edmonton, Alberta, Canada) using sterile conditions. After ethanol evaporation, the fatty acids were resuspended in FCS (volume equating to 5 % of the final volume of medium being prepared) and incubated in a shaking water bath at 37 °C for 1 h, with vortexing every 15 minutes. After 1 hour, 10 mL of IMDM or MEM was added to each tube and incubated for a further 30 min at 37 °C. The fatty acid-containing media was adjusted to the final volume with IMDM or MEM and used immediately. In some experiments, aliquots of a vitamin E (dl-α-tocopherol; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) stock solution (67 mg/mL in ethanol) were added to the fatty acid-supplemented medium to determine if the effects of EPA and DHA were related to lipid oxidation products formed in culture. The ethanol concentration in the media did not exceed 0.07 % v/v.

Growth experiments

Preliminary experiments were conducted with MDA-MB-231 and MCF-7 cells to determine an optimal concentration of LA to stimulate growth (75 µM) and appropriate concentrations of EPA and DHA to inhibit cell growth in the absence and presence of LA (60 µM EPA + 40 µM DHA, and 45 µM EPA + 30 µM DHA + 75 µM LA, respectively), relative to untreated cells. The addition of LA to 60 µM EPA + 40 µM DHA was found to be toxic to the cells, thus EPA and DHA at 45 µM and 30 µM, respectively, were used in combination with 75 µM LA, and inhibited growth to a similar extent as that observed with 60 µM EPA + 40 µM DHA. An additional control LA treatment (150 µM) was added to each experiment to ensure that the total fatty acid concentration used was not cytotoxic to cells. In some experiments, MDA-MB-231 cells were exposed to either 100 µM EPA or 100 µM DHA, ± 50 or 100 µM vitamin E, to examine the relative potency of EPA versus DHA, and to examine whether the addition of vitamin E to the culture media affected n-3 PUFA-induced growth inhibition.

Cells were seeded at 1 x 10^6 cells per flask (75 cm^2) in 25 mL of medium containing 5 % v/v FCS and allowed 48 h to adhere. In most experiments, duplicate flasks were seeded for each fatty acid treatment. (In this thesis, one 'experiment' refers to a set of flasks seeded on the same day and from the same cell passage.) After 48 h, the culture medium was replaced with fresh medium containing the experimental fatty acids (as described) and the cells were further incubated for 72 h, during which time the medium was not changed. After 72 h, the cells were harvested using trypsin-EDTA (Gibco Invitrogen Corporation, Burlington, Ontario, Canada), and viable cells were counted under a microscope by trypan blue (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) exclusion. Cell counts for each fatty acid treatment were converted to a percentage of the control treatment, 75 µM LA.

[Methyl-^{3}H]-thymidine incorporation

The rate of DNA synthesis following fatty acid treatment was estimated by [methyl-^{3}H]-thymidine incorporation. Cells were seeded at 2 x 10^4 cells/mL/well into sterile, 24-well flat-bottom tissue culture plates (Fisher Scientific, Edmonton, Alberta, Canada) and exposed to fatty acids as described for the 'Growth experiments'. Four hours prior to harvesting, the cells were pulsed with [methyl-^{3}H]-thymidine (2.5 μCi per well) (Amersham Biosciences, Baie D'Urfe, Quebec, Canada). To harvest the cells, the medium was gently removed from each well and discarded, and cells were incubated with 300 μL of trypsin-EDTA for 3 minutes at room temperature. Phosphate-buffered saline (PBS) (600 μL) was then added to each well and mixed thoroughly to evenly suspend the cells. Aliquots (200 μL in quadruplicate) were transferred to 96-well round-bottom plates (Costar®, Fisher Scientific, Edmonton, Alberta, Canada). The cells were harvested onto Unifilter-96 GF/C microplates (Packard Bioscience, Mississauga, Ontario, Canada) with a Packard FilterMate™ harvester. Plates were counted on a TopCount® NXT Microplate Scintillation and Luminescence Counter (Packard Bioscience, Mississauga, Ontario, Canada) after the addition of Microscint™ O-Cocktail (Packard Bioscience, Mississauga, Ontario, Canada). Disintegrations per minute (DPM) were corrected for final cell number in each fatty acid treatment using the corresponding flask cell count data from parallel growth experiments. DPMs were then expressed as a percentage of the control treatment, 75 μM LA.

Western blot analysis for PCNA, PRK, and Akt/phospho-Akt

For analysis of Akt and phospho-Akt protein expression, fatty acid-treated MDA-MB-231 cells were stimulated with 50 ng/mL epidermal growth factor (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) for 30 minutes at 37 °C prior to harvesting with trypsin-EDTA. Whole cell protein lysates were prepared from fatty-acid treated cells by the addition of lysis buffer (100-250 μL) consisting of 20 mM TrisHCl pH 7.4, 137 mM NaCl, 10 % v/v glycerol, 1 % v/v Nonidet P-40, and 2 mM EDTA with freshly added protease and phosphatase inhibitor cocktails (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) diluted 1:100. Lysates were incubated on ice for 30 minutes, then centrifuged at 4 °C for 15 minutes at 12,000 rpm, and supernatants were aliquotted and stored at –80 °C. The protein concentration of cell lysates was determined using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Equal amounts of protein from each treatment were separated by SDS-PAGE on 10 % polyacrylamide gels. Rainbow™ (Amersham Biosciences, Baie D'Urfe, Quebec, Canada) colored molecular weight standards were used to monitor protein separation. Proteins were electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Baie D'Urfe, Quebec, Canada). Even protein loading and transfer was confirmed by staining with Ponceau S (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Membranes were blocked for one hour at room temperature with TBST (10 mM TrisHCl pH 7.4, 150 mM NaCl, 0.1 % v/v Tween-20) and 5 % w/v powdered milk. Primary antibodies to PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) and PRK (BD Pharmingen™, Mississauga, Ontario, Canada) were diluted 1:2000 and 1:1000, respectively, in TBST containing 1 % w/v powdered milk and incubated with membranes for one hour at room temperature. Parallel blots were probed under the same conditions with primary antibody for β-actin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) diluted 1:2000 to confirm even protein loading. The primary antibodies to phospho-Akt (Ser473) and Akt (Cell Signaling Technology, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) were diluted 1:500 and 1:3000, respectively, in TBST containing 5 % w/v bovine serum albumin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and incubated with membranes overnight at 4 °C. All membranes were incubated with secondary antibodies diluted in TBST containing 1 % w/v powdered milk for one hour at room temperature. For PCNA, PRK and β-actin, HRP-conjugated anti-mouse secondary antibody (BD Pharmingen™, Mississauga, Ontario, Canada) was used at 1:5000 dilution, and for phospho-Akt and Akt, HRP-conjugated anti-rabbit secondary antibody (BD Transduction Laboratories™, Mississauga, Ontario, Canada) was used at 1:500 dilution. Membranes were developed using an enhanced chemiluminescence (ECL™) detection kit (Amersham Biosciences, Baie D'Urfe, Quebec, Canada) and Hyperfilm™ ECL™ film (Amersham Biosciences, Baie D'Urfe, Quebec, Canada). The relative intensities of band signals were determined using a Bio-Rad GS-670 imaging densitometer (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada).

Cyclin protein expression

Cells were harvested from flasks and counted as described. Cells were washed once in wash buffer (PBS containing 1 % v/v FCS), and then fixed by adding 5 mL cold (-20 °C) 100 % methanol, dropwise, while vortexing to prevent cell clumping. Cells were incubated at -20 °C for a minimum of overnight, and not longer than 30 days, prior to cyclin analysis. Just prior to staining, methanol was removed from the samples by centrifugation at 250 x g for 10 min. Cells were washed once in wash buffer, and then permeabilized in 5 mL of cold 0.25 % v/v Triton X-100 (VWR, Edmonton, Alberta, Canada) in wash buffer for 5 min on ice. Cells were washed once in wash buffer, and distributed at approximately 1 x 10^6 cells/tube into 12 x 75 mm round bottom tubes (Falcon™, VWR, Edmonton, Alberta, Canada) for staining. The cells were incubated in the dark for 30 min at room temperature with 0.5 μg of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibodies specific for cyclins D1 and B1, or FITC-conjugated IgG1 isotype-specific antibody as a control (BD Pharmingen™, Mississauga, Ontario, Canada). After incubation, cells were washed once in 2 mL of wash buffer and resuspended in 0.5 mL of PBS. Cellular fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) and analyzed using Cell Quest software (Becton Dickinson, Sunnyvale, CA). Gates were set with fluorescence intensity above that of the isotype control to calculate the percent of cyclin-positive cells.

Caspase activity

Activated caspases were detected using a Caspase Detection Kit (FITC-VAD-FMK) from Oncogene (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Fatty acid-treated cells were harvested from the flasks and 5 x 10^5 cells per treatment were incubated with 1 μL of the FITC-VAD-FMK caspase inhibitor (which irreversibly binds to the active site of active caspase enzymes) for one hour at 37 °C. Cells were washed twice with incubation buffer (provided with the kit) and cell fluorescence was detected by flow cytometry using the flow cytometer described above.

Mitochondrial membrane potential

Changes in mitochondrial membrane potential in the tumor cells were detected by a MitoCapture™ Apoptosis Detection kit (Calbiochem, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). This kit utilizes a cationic dye that exhibits distinct fluorescence in healthy cells versus apoptotic cells. In healthy cells, the MitoCapture™ reagent accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, the MitoCapture™ reagent cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential, and thus remains in the cytoplasm in its monomer form, generating a green fluorescence. Fatty acid-treated cells were harvested from the flasks and 5 x 10^5 cells per treatment were incubated with 1 mL of diluted MitoCapture™ reagent for 20 minutes at 37 °C. The cells were pelleted, resuspended in incubation buffer (provided with the kit) and cell fluorescence was detected immediately by flow cytometry using the flow cytometer described above.

DNA fragmentation

The Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) was used to quantify mono- and oligonucleosomes in cytoplasmic lysates prepared from fatty-acid treated cells, according to the manufacturer's instructions. Briefly, 2 x 10^4 cells were lysed in 200 µL of lysis buffer for 30 minutes at room temperature. Lysates were centrifuged for 10 minutes at 200 x g, after which 20 µL aliquots of supernatant were pipetted into a streptavidin-coated microplate, along with 80 µl per well of the immunoreagent (biotin-labeled anti-histone monoclonal antibody and peroxidase-conjugated anti-DNA monoclonal antibody). The microplate was incubated on a plate shaker (300 rpm) for 2 hours at room temperature, then wells were washed and 100 µL of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate solution was added to each well, and incubated for approximately 15 minutes. Absorbance was measured at 405 nm on a Molecular Devices SpectraMax 190 spectrophotometer (Sunnyvale, CA).

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release was measured using the CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega Corporation, Madison, WI). Briefly, cells were grown in 24-well plates and treated with the experimental fatty acids as described for [methyl-^{3}H]-thymidine incorporation assessment. After 72 h of fatty acid treatment, the media was gently removed from each well, spun at 250 x g to remove any cells, and then 50 µL aliquots were transferred into a 96-well flat-bottom plate (Corning Incorporated, Corning, NY). The Reaction Mixture provided with the kit was then added (50 µL per well), and the plate was incubated in the dark at room temperature for 30 min. Stop solution (50 µL of 1 M acetic acid) was then added to each well, and the absorbance read immediately at 492 nm in a Molecular Devices SpectraMax 190 spectrophotometer (Sunnyvale, CA).

Tumor cell phospholipid fatty acid analysis

Lipids were extracted from fatty acid-treated cells by a modified Folch (Folch *et al.*, 1957) procedure (Field *et al.*, 1988). Individual phospholipids were separated from the lower phase on thin layer chromatography plates (HPK silica gel 60 nm 10 x 10 cm, Whatman, Clifton, NJ) using a solvent system consisting of chloroform : methanol : 2-propanol : 0.25 % w/v KCl : triethylamine (30:9:25:6:18 by volume) as previously described (Touchstone *et al.*, 1980). Separated phospholipids were visualized with 8-anilino-1-naphthalene-sulfonic acid (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and identified under ultraviolet light with appropriate standards (Supelco Canada Ltd., Oakville, Ontario, Canada). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM) fatty acid methyl esters were prepared from the scraped silica band using 14 % w/v BF_3/methanol reagent (Morrison and Smith, 1964) and separated by automated gas liquid chromatography (Varian CP-3800, Varian Inc., Mississauga, Ontario, Canada) on an SGE BP20 column (60 m x 0.25 mm internal diameter; Varian Inc., Mississauga, Ontario, Canada). The analytical conditions used separate all saturated, mono-, di-, and polyunsaturated fatty acids from 14 to 24 carbons in chain length.

Electrophoretic mobility shift assay for NFκB

Nuclear extracts were prepared from fatty acid treated cells by incubation for 15 minutes at 4 °C in hyposmotic lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.625 % v/v Nonidet P-40, 3 mM dithiothreitol) plus freshly added protease inhibitor cocktail (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Lysates were centrifuged for one minute at 15,000 rpm, and the nuclear pellet was resuspended in hyperosmotic buffer (20 mM HEPES, 0.42 M NaCl, 5 mM EDTA, 10 % v/v glycerol, 5 mM DTT) plus freshly added protease inhibitor cocktail for 30 minutes at 4 °C. Samples were centrifuged at 15,000 rpm for 10 minutes, and nuclear extracts were aliquotted and frozen at –80 °C. Protein concentrations of nuclear extracts were assayed by BCA assay as described above. Electrophoretic mobility shift assays were performed as previously described (Jijon *et al.*, 2002). Briefly, labeling of NFκB site oligonucleotide [5'-AGTTGAGGGGACTTTCCCAGGC-3' (Santa Cruz Biotechnology, Santa Cruz, CA)] was performed using T4 polynucleotide kinase (Gibco Invitrogen Corporation, Burlington, Ontario, Canada) following the manufacturer's instructions. Nuclear extracts (5 μg) were incubated at room temperature with labeled oligonucleotides and were separated by electrophoresis in a 6 % TBE (Tris base, boric acid, EDTA) acrylamide gel. Gels were dried for 1 h in a gel drier (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada) and autoradiography was conducted at –70 °C with Kodak X-OMAT film. NFκB DNA binding activity in the same nuclear lysates was confirmed using the ELISA-based TransAM™ NFκB Family Transcription Factor Assay Kit (Active Motif, MJS BioLynx Inc., Brockville, Ontario, Canada) according to the manufacturer's instructions. The relative DNA binding activity of both p50- and p65-containing NFκB was determined using the p50- and p65-specific antibodies supplied with the kit. Specificity of NFκB binding was checked by measuring the ability of soluble wild-type or mutated consensus oligonucleotides to inhibit binding of both p50- and p65-containing NFκB.

Inhibition of NFκB through proteolysis inhibition

The proteasome inhibitor MG-132 was purchased from Calbiochem (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). MDA-MB-231 and MCF-7 cells were seeded at 2 x 10^6 cells/flask and 1 x 10^6 cells/flask, respectively, in medium containing 5 % v/v FCS, and allowed 48 hours to adhere. After 48 h, the culture medium was replaced with fresh medium containing MG-132 (0.1 to 10 μM) in DMSO. The DMSO concentration in the media did not exceed 0.1 % v/v. Untreated and DMSO-treated cells were cultured in parallel as controls. After 48 h, the cells were harvested using trypsin-EDTA (Gibco Invitrogen Corporation, Burlington, Ontario, Canada), and both viable and non-viable cells were counted under a microscope by trypan blue (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) exclusion. Cell viability was calculated as [number of viable cells/(number of viable + non-viable cells) x 100].

Statistical analysis

All statistical analyses were conducted using the SAS statistical package, version 8 (SAS Institute, Cary, NC). Data sets that were not normally distributed were log-transformed prior to statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA), blocked by experiment/passage number, followed by a Tukey test to identify significant ($p<0.05$) differences between fatty acid treatments.

3.3 RESULTS

EPA and DHA decrease the population growth of human breast cancer cells

Treatment of MDA-MB-231 and MCF-7 cells with n-3 PUFA resulted in a significantly lower number of viable cells after 72 hours compared to control-treated cells (Figure 3-1). A mixture of EPA and DHA (60 μM EPA + 40 μM DHA) significantly ($p<0.05$) decreased MDA-MB-231 cell population growth by 51 %, and MCF-7 cell growth by 46 %, compared to the control treatment, 75 μM LA. Incubation of cells with n-3 PUFA in the presence of LA (i.e. 45 μM EPA + 30 μM DHA + 75 μM LA) significantly ($p<0.05$) decreased the number of viable MDA-MB-231 and MCF-7 cells by 46 % compared to incubation with 75 μM LA alone. Both n-3 PUFA treatments significantly ($p<0.05$) decreased viable cell numbers compared to untreated cells in both cell lines, confirming that EPA and DHA have specific growth inhibitory effects that are not due to LA deprivation. The decrease in cell population growth also was not due to a cytotoxic effect of increased fatty acid concentration in the media (150 μM) as incubation with 150 μM LA, compared to 75 μM LA, did not significantly affect growth.

In additional experiments, MDA-MB-231 cells were exposed to either 100 μM EPA or 100 μM DHA, or a combination of 75 μM EPA + 75 μM LA or 75 μM DHA +

75 μM LA, in order to examine the relative potency of each n-3 PUFA. Both EPA and DHA, either alone or in the presence of 75 μM LA, significantly ($p<0.05$) inhibited cell population growth as estimated by viable cell counts (Figure 3-2). DHA, at a concentration of 100 μM, was found to be more effective ($p<0.05$) at inhibiting the growth of MDA-MB-231 cells (viable cells were reduced by 65 %) compared to 100 μM EPA (viable cells were reduced by 42 %).

N-3 PUFA-induced growth inhibition is not abrogated by vitamin E

In additional experiments, MDA-MB-231 cells were exposed to either 100 μM EPA or 100 μM DHA ± 50 or 100 μM vitamin E, to determine whether the observed n-3 PUFA-induced growth inhibition was related to the formation of lipid oxidation products. The addition of either 50 or 100 μM vitamin E did not abrogate the growth-inhibitory effects of EPA and DHA, as the numbers of viable cells obtained following treatment with either 100 EPA or 100 μM DHA were not changed with the addition of vitamin E (Figure 3-3). Neither 100 μM vitamin E nor vehicle treatment (0.06 % v/v ethanol) alone affected cell growth.

EPA and DHA decrease proliferation of human breast cancer cells

To assess whether n-3 PUFA alter tumor cell proliferation, we examined the effects of EPA and DHA on [methyl-^{3}H]-thymidine incorporation and expression of the proliferation markers PCNA and PRK. In the MDA-MB-231 cell line, both n-3 PUFA treatments (i.e. 60 μM EPA + 40 μM DHA and 45 μM EPA + 30 μM DHA + 75 μM LA) significantly ($p<0.05$) decreased [methyl-^{3}H]-thymidine incorporation compared to both 75 μM LA-treated and untreated cells (Figure 3-4), indicating a lower rate of DNA synthesis following exposure to n-3 PUFA. There was no significant effect of 150 μM LA on [methyl-^{3}H]-thymidine incorporation in this cell line compared to 75 μM LA. Similarly, both n-3 PUFA treatments significantly ($p<0.05$) decreased [methyl-^{3}H]-thymidine incorporation compared to 75 μM LA-treated or untreated cells in the MCF-7 cell line (Figure 3-4). Compared to 75 μM LA treatment, [methyl-^{3}H]-thymidine incorporation was lower when treated with 150 μM LA in this cell line, but this difference did not reach statistical significance.

The protein levels of both PCNA and PRK, as measured by western blotting, were significantly reduced by both n-3 fatty acid treatments in both cell lines, with the exception of PCNA in MCF-7 cells (Figure 3-5), supporting decreased tumor cell proliferation with n-3 PUFA treatment.

EPA and DHA do not significantly decrease the percentage of cyclin D1- and cyclin B1-positive human breast cancer cells

There was a trend toward lower numbers of cyclin D1- and cyclin B1-positive MDA-MB-231 cells with n-3 PUFA treatment, but this effect did not reach statistical significance (Figure 3-6A). There were no differences in the percentages of cyclin D1- or cyclin B1-positive MCF-7 cells among fatty acid treatments (Figure 3-6B).

EPA and DHA induce apoptosis in human breast cancer cells

To determine whether EPA and DHA induced apoptosis of the tumor cells, we examined the effects of EPA and DHA on markers of apoptosis, specifically caspase protein activity, mitochondrial membrane potential, and DNA fragmentation. The effects of n-3 PUFA on caspase protein activity are shown in Figure 3-7. In the MDA-MB-231 cell line, treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA significantly ($p<0.05$) increased the percentage of cells with activated caspases by 29 % and 22 %, respectively, compared to the 75 μM LA and 150 μM LA control treatments. There was no significant effect of n-3 PUFA on the percentage of MCF-7 cells with active caspases (Figure 3-7).

The effects of n-3 PUFA on mitochondrial membrane potential (MMP) are shown in Figure 3-8. In both cell lines, treatment with 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA significantly ($p<0.05$) increased the percentage of cells with a loss of mitochondrial membrane potential compared to the control treatment, 75 μM LA. The percentage of cells with a loss of MMP in the 150 μM LA treatment did not differ significantly from the 75 μM LA treatment for either cell line.

Treatment with n-3 PUFA induced significant DNA fragmentation in both cell lines as determined by the Cell Death Detection ELISA^{PLUS} (Table 3-1). In both cell lines, treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA significantly ($p<0.05$) increased the absorbance values (reflecting mono- and oligonucleosomes in the cytoplasmic cell lysates) relative to LA-treated cells. In contrast, treatment with 150 μM LA did not significantly increase DNA fragmentation compared to either 75 μM LA-treated or untreated cells in either cell line.

The increased caspase activity, loss of mitochondrial membrane potential, and increased DNA fragmentation observed with n-3 PUFA treatment all provide support for the induction of tumor cell apoptosis by EPA and DHA.

EPA and DHA do not increase necrosis in human breast cancer cells

There were no significant differences in LDH release after 72 h incubation with the various fatty acids for either cell line (Table 3-2). Similarly, the proportion of PI-positive MDA-MB-231 cells following treatment with n-3 PUFA did not exceed 10 %, compared to 4 to 7 % PI-positive cells treated with LA (Figure 3-9). These observations suggest that in these human breast cancer cell lines, n-3 PUFA treatment does not markedly affect plasma membrane integrity, which is a hallmark of necrosis.

EPA and DHA are significantly incorporated into membrane phospholipids

The fatty acid composition of PC, PE, PI, PS and SM of PUFA-treated MDA-MB-231 and MCF-7 cells is shown in Tables 3-3 (MDA-MB-231) and 3-4 (MCF-7). Below is a detailed description of the results.

Phosphatidylcholine. Concentrations of EPA (C20:5n-3), DHA (C22:6n-3), docosapentaenoic acid (DPA, C22:5n-3), and total n-3 PUFA were significantly ($p<0.05$) increased in both cell lines following treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA, as compared to treatment with either 75 or 150 μM LA (Tables 3-3 and 3-4). Enrichment of EPA, DPA, DHA and total n-3 PUFA was significantly lower with 45 μM EPA + 30 μM DHA + 75 μM LA versus 60 μM EPA + 40 μM DHA treatment in both cell lines. Cells treated with 60 μM EPA + 40 μM DHA, but not 45 μM EPA + 30 μM DHA + 75 μM LA, had a significantly lower concentration of LA (C18:2n-6) compared to treatment with 75 μM LA in both cell lines. Treatment with 150 μM LA significantly increased the concentration of LA compared to treatment with 75 μM LA in both cell lines. Treatment with 60 μM EPA + 40 μM DHA, 45 μM EPA + 30 μM DHA + 75 μM LA, or 150 μM LA all significantly decreased the concentration of AA (C20:4n-6) as compared to treatment with 75 μM LA in both MDA-MB-231 and MCF-7 cells. In both cell lines, the total n-6 PUFA concentration was significantly lower with 60 μM EPA + 40 μM DHA treatment and significantly higher with 150 μM LA treatment, relative to 75 μM LA-treated cells. In both cell lines, the total SFA concentration was increased, whereas the total PUFA concentration was decreased, with 60 μM EPA + 40 μM DHA treatment relative to 75 μM LA treatment.

Phosphatidylethanolamine. Concentrations of EPA, DHA, DPA, and total n-3 PUFA were significantly increased in both cell lines following treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA, as compared to treatment with either 75 or 150 μM LA (Tables 3-3 and 3-4). Enrichment of EPA, DPA, DHA and total n-3 PUFA was significantly lower with 45 μM EPA + 30 μM DHA + 75 μM LA versus 60 μM EPA + 40 μM DHA treatment in both cell lines. MDA-MB-231 cells treated with 60 μM EPA + 40 μM DHA had a significantly lower concentration of LA as compared to 75 μM LA-treated cells. MCF-7 cells treated with either n-3 PUFA treatment had a significantly lower LA concentration as compared to treatment with 75 μM LA. Both n-3 PUFA treatments significantly decreased the AA concentration in MDA-MB-231 cells, whereas 45 μM EPA + 30 μM DHA + 75 μM LA and 150 μM LA decreased the AA concentration in MCF-7 cells, relative to cells treated with 75 μM LA. In both cell lines, the total n-6 PUFA concentration was significantly decreased with both n-3 PUFA treatments relative to 75 μM LA treatment. However, the total n-6 PUFA concentration was significantly higher with 45 μM EPA + 30 μM DHA + 75 μM LA versus with 60 μM EPA + 40 μM DHA treatment. In general, the highest total n-3 PUFA concentrations were seen in this phospholipid in both cell lines, with n-3 PUFA comprising up to 41 to 43 % of the total fatty acids.

Phosphatidylinositol. Concentrations of EPA, DHA, DPA, and total n-3 PUFA were significantly increased in both cell lines following treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA, as compared to treatment with either 75 or 150 μM LA (Tables 3-3 and 3-4). Enrichment of EPA, DPA, DHA and total n-3 PUFA was significantly lower with 45 μM EPA + 30 μM DHA + 75 μM LA versus 60 μM EPA + 40 μM DHA treatment in both cell lines. Treatment with 60 μM EPA + 40 μM DHA, but not 45 μM EPA + 30 μM DHA + 75 μM LA, resulted in a significantly lower concentration of LA compared to treatment with 75 μM LA in both cell lines. Both n-3 PUFA treatments significantly decreased the AA concentration in both cell lines as compared to 75 μM LA treatment, whereas 150 μM LA treatment also decreased the AA concentration in MCF-7 cells, relative to cells treated with 75 μM LA. In both cell lines, the total n-6 PUFA concentration was significantly decreased with both n-3 PUFA treatments relative to 75 μM LA treatment. However, the total n-6 PUFA concentration was significantly higher with 45 μM EPA + 30 μM DHA + 75 μM LA versus with 60 μM EPA + 40 μM DHA treatment. In general, the AA concentration was highest in this phospholipid relative to the other phospholipids in both cell lines.

Phosphatidylserine. Concentrations of EPA, DHA, DPA, and total n-3 PUFA were significantly increased in both cell lines following treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA, as compared to treatment with either 75 or 150 μM LA (Tables 3-3 and 3-4). Enrichment of EPA, DPA, DHA and total n-3 PUFA was significantly lower with 45 μM EPA + 30 μM DHA + 75 μM LA versus 60 μM EPA + 40 μM DHA treatment in both cell lines. Treatment with 60 μM EPA + 40 μM DHA, but not 45 μM EPA + 30 μM DHA + 75 μM LA, resulted in a significantly lower concentration of LA compared to treatment with 75 μM LA in both cell lines. Both n-3 PUFA treatments significantly decreased the AA concentration in MDA-MB-231 cells, whereas 45 μM EPA + 30 μM DHA + 75 μM LA and 150 μM LA decreased the AA concentration in MCF-7 cells, relative to cells treated with 75 μM LA. The total n-6 PUFA concentration was significantly decreased with 60 μM EPA + 40 μM DHA treatment in both cell lines, and with 45 μM EPA + 30 μM DHA + 75 μM LA treatment in MDA-MB-231 cells only, relative to 75 μM LA treatment. In both cell lines, the total SFA concentration was increased, whereas the total PUFA concentration was decreased, with 60 μM EPA + 40 μM DHA treatment relative to 75 μM LA treatment.

Sphingomyelin. In general, the total SFA concentration was much higher and the total n-3 and n-6 PUFA concentrations were much lower in this phospholipid relative to the other phospholipids in both cell lines. The total n-3 PUFA concentration was significantly increased with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA treatment in MCF-7 cells, but only with 60 μM EPA + 40 μM DHA treatment in MDA-MB-231 cells, as compared to treatment with 75 μM LA (Tables 3-3 and 3-4). In MCF-7 cells only, the LA and total n-6 PUFA concentrations were significantly lower with 60 μM EPA + 40 μM DHA treatment, compared to treatment with 75 μM LA.

EPA and DHA decrease Akt phosphorylation and NFκB DNA-binding activity

The decreased tumor cell proliferation and survival observed in this study with n-3 PUFA may be caused by altered signaling through cell survival signaling pathways. We examined the effects of EPA and DHA on the phosphorylation state (Ser473) of Akt (Figure 3-10). Both n-3 PUFA treatments decreased the phosphorylation of Akt (Ser473), compared to the 75 μM LA treatment, in both cell lines. This decrease in phosphorylated protein did not appear to be due to a decrease in total Akt protein levels, as these remained unchanged by fatty acid treatment.

Since Akt plays a role in regulating the activity of the NFκB transcription factor, we hypothesized that a decrease in the activated form of Akt induced by EPA and DHA treatment may also lead to a decrease in NFκB DNA binding activity. As indicated in Figure 3-11A, both n-3 PUFA treatments decreased the DNA binding activity of NFκB, but only in MDA-MB-231 cells. These results were confirmed by the use of an ELISA kit that detects and quantifies NFκB DNA binding, which demonstrated that the DNA binding activity of both p50- and p65-containing NFκB was significantly impacted by n-3 PUFA treatment (Figure 3-11B). DNA binding activity of NFκB was somewhat higher in cells treated with 45 μM EPA + 30 μM DHA + 75 μM LA versus 60 μM EPA + 40 μM DHA. NFκB binding was inhibited by the addition of wild-type, but not mutated, oligonucleotide of NFκB binding sequence, confirming the specificity of NFκB binding in this assay (Figure 3-11B). Changes in NFκB DNA-binding activity with n-3 PUFA treatment could not be detected in MCF-7 cells.

Inhibition of proteolysis inhibits the growth of human breast cancer cells

To determine whether pharmacological inhibition of NFκB activity could mimic the effects of n-3 PUFA and affect the growth of human breast cancer cells, MDA-MB-231 and MCF-7 cells were treated with 0.1 to 10 μM of the proteasome inhibitor MG-132. As is apparent in Figure 3-12, MG-132 treatment decreased cell viability (Figure 3-12A) as well as the total number of cells (Figure 3-12B) of both cell lines, indicating that tumor cell survival and growth was reduced by MG-132. Notably, the MDA-MB-231 cell line was much more sensitive to growth inhibition by MG-132, as the MG-132 inhibitor had to be diluted approximately 10-fold in order to achieve similar decreases in cell number and viability as that seen in the MCF-7 cell line.

3.4 DISCUSSION

We have shown that a mixture of EPA and DHA significantly inhibited the population growth (or expansion) of MDA-MB-231 and MCF-7 breast cancer cells as estimated by a decrease in viable cell numbers, as compared to either untreated or LA-treated cells. This finding is in agreement with the existing literature demonstrating that n-3 PUFA inhibit breast cancer cell growth *in vitro* (Begin *et al.*, 1986; Begin *et al.*, 1988; Rose and Connolly, 1990; Grammatikos *et al.*, 1994b; Chajes *et al.*, 1995; Noguchi *et al.*, 1995; Abdi-Dezfuli *et al.*, 1997; Senzaki *et al.*, 1998; Yamamoto *et al.*, 1999; Chamras *et al.*, 2002). However, the present study was able to extend these earlier findings by demonstrating that significant tumor growth inhibition by n-3 PUFA could be achieved in the presence of abundant linoleic acid in the medium. To our knowledge, only one other *in vitro* study has provided LA in combination with n-3 PUFA to breast cancer cells (Noguchi *et al.*, 1995), using a concentration (625 ng/mL, or ~2.2 μM) of LA well below the amount used in our study (75 μM), and below the concentration of LA in medium prepared with 5 % v/v FCS (3 μM LA; Ma *et al.*, 2002). Our data supports the hypotheses that tumor growth inhibition observed with n-3 PUFA treatment: 1) is a specific effect of the n-3 PUFA, and 2) is not the result of deficient LA in the medium, as

EPA and DHA significantly decreased the growth of MDA-MB-231 and MCF-7 cells compared to that of untreated cells, and significant growth inhibition was still induced by n-3 PUFA when provided in combination with LA. Thus, we have demonstrated that n-3 PUFA have specific growth-inhibitory, apoptosis-promoting effects on breast cancer cells *in vitro* even when provided with sufficient LA in the media.

The MDA-MB-231 and MCF-7 cell lines differ in their growth characteristics and representative disease stage; more specifically, the MDA-MB-231 cell line is estrogen receptor-negative and is highly metastatic, whereas the MCF-7 cell line is growth-responsive to estrogen and is poorly metastatic (Dickson *et al.*, 1986; Timoshenko *et al.*, 2003). The finding that the growth of both of these two phenotypically dissimilar cell lines is inhibited by n-3 PUFA suggests that the effects of n-3 PUFA are not limited to one particular cell line but may be relevant to a broader spectrum of mammary tumors.

Studies that have attempted to dissect the individual effects of EPA versus DHA on various breast cancer cell lines have yielded inconsistent results. However, the findings of our study are consistent with other studies involving the MDA-MB-231 cell line, which suggest that DHA is more potent than EPA in inhibiting breast tumor cell growth (Rose and Connolly, 1990; Chajes *et al.*, 1995; Noguchi *et al.*, 1995). Although there are differing reports regarding the potency of EPA and DHA in other breast cancer cell lines (Begin *et al.*, 1986; Begin *et al.*, 1988; Chajes *et al.*, 1995; Abdi-Dezfuli *et al.*, 1997), our results demonstrate that at equal molar concentrations, DHA is more potent than EPA with respect to inhibiting the growth of the MDA-MB-231 cell line. This observation will be of importance in planning animal or human feeding trials.

Many mechanisms have been proposed to explain the growth-inhibitory effects of n-3 PUFA on breast cancer cells, but few of the published studies have attempted to determine whether EPA and DHA decrease cell proliferation or increase cell death, or both. Among the *in vitro* reports, only one study has reported an effect of n-3 PUFA on breast cancer cell cycle distribution, which demonstrated that DHA caused a cell cycle arrest of MCF-7 cells (Kachhap *et al.*, 2001). Begin *et al.* attributed the effects of EPA and DHA to cytotoxic (necrotic) cell death (Begin *et al.*, 1985; Begin *et al.*, 1986; Begin *et al.*, 1988), whereas only one study has documented n-3 PUFA-induced apoptosis in human breast cancer cells *in vitro* (Yamamoto *et al.*, 1999). Among the animal studies, decreased mammary tumor growth has been attributed to "tumor cell loss" (Gabor and Abraham, 1986), prolonged DNA synthesis (Istfan *et al.*, 1995), decreased tumor cell proliferation (Senzaki *et al.*, 1998), and to a combination of decreased proliferation and apoptotic cell death (Connolly *et al.*, 1999).

In the present study, we observed a decrease in [methyl-^{3}H]-thymidine uptake, as well as decreases in the expression of the proliferation-related proteins, PCNA and PRK, indicating that proliferation of breast cancer cells is impaired by n-3 PUFA treatment. In addition, the increase in DNA fragmentation, loss of mitochondrial membrane potential, and increased activity of caspase proteins observed in the present study all support the hypothesis that n-3 PUFA also induce apoptotic cell death. These findings demonstrate that n-3 PUFA both impair proliferation and induce apoptosis of breast cancer cells and that this does not appear to be the result of insufficient LA. Inconsistent with our expectations, we did not see an effect of n-3 PUFA on caspase activation in the MCF-7 cell line. Kottke *et al.* (2002) reported a lack of correlation between caspase activation (as measured by cleavage of procaspases-9 and –7 and digestion of PARP and ICAD) and caspase activity assays (e.g. cleavage of tetrapeptide substrates such as DEVD-AFC, LEHD-AFC, VEID-AFC), and suggested that a sequestration of cleaved caspases within the cell may result in an underestimation of caspase activity with the latter assays. This observation might explain the lack of caspase activity observed in MCF-7 cells in our study.

The addition of LA to EPA and DHA appears to decrease [methyl-^{3}H]-thymidine uptake, but not viable cell number, to a greater extent than EPA and DHA treatment alone. However, these two measures can not be compared directly as the cell seeding density for the [methyl-^{3}H]-thymidine uptake assay was half that of the growth experiments conducted in flasks. We and others (Begin *et al.*, 1985; Begin *et al.*, 1986) have observed that growth inhibition induced by n-3 PUFA is sensitive to cell density, and we suspect that growth inhibition induced by the n-3 PUFA within the incubation period may have differed between the 24-well plates (used to assess [methyl-^{3}H]-thymidine uptake) and the flasks (used to count viable cells). Alternatively, it is possible that the addition of LA to EPA and DHA somehow exaggerates the effect of n-3 PUFA on cell proliferation, as the addition of LA to 60 μM EPA + 40 μM DHA proved toxic to the cells, requiring us to use lower concentrations of EPA and DHA when in combination with LA to obtain a similar rate of growth inhibition. This observation could be important *in vivo*, and requires further study.

Several studies have suggested that the growth-inhibitory effects of n-3 PUFA on breast cancer cells are the result of increased lipid peroxidation (Begin *et al.*, 1985; Begin *et al.*, 1988; Gonzalez *et al.*, 1991; Gonzalez *et al.*, 1993; Chajes *et al.*, 1995). This is unlikely the mechanism in the current study, as the addition of 50 or 100 μM vitamin E (dl-α-tocopherol) to the culture medium did not affect the growth inhibition induced by either 100 μM EPA or 100 μM DHA in MDA-MB-231 cells.

The present study demonstrated that in both cell lines, EPA and DHA were significantly incorporated into membrane phospholipids following treatment with either n-3 PUFA preparation. In addition, the concentration of DPA (C22:5n-3) was also significantly increased (in PC, PE and PI) with either n-3 PUFA treatment, suggesting that interconversion of the long-chain n-3 PUFA had occurred. Enrichment of n-3 PUFA was lower in cells treated with 45 μM EPA + 30 μM DHA + 75 μM LA than in cells treated with 60 μM EPA + 40 μM DHA. This was not unexpected due to the lower concentration of n-3 PUFA in the 45 μM EPA + 30 μM DHA + 75 μM LA treatment as well as the competition of EPA and DHA with LA for membrane phospholipid incorporation. For the most part, enrichment of n-3 PUFA in membrane phospholipids did not occur at the expense of LA in either cell line, as treatment with 45 μM EPA + 30 μM DHA + 75 μM LA did not decrease the concentration of LA relative to that observed with 75 μM LA treatment alone. In both cell lines, both n-3 PUFA treatments significantly decreased the AA concentration in PI relative to 75 μM LA-treated cells. The same effect was observed in PC, PE and PS in MDA-MB-231 cells. However, in the MCF-7 cell line, significant decreases in AA concentrations were observed primarily with the 150 μM LA and 45 μM EPA + 30 μM DHA + 75 μM LA treatments. AA concentrations were very low in MCF-7 cells, despite significant LA concentrations in phospholipids of cells incubated with LA. This likely reflects the described $^{\Delta}$6-desaturase deficiency in the MCF-7 cell line (Grammatikos *et al.*, 1994a; Grammatikos *et al.*, 1994b). These observations would suggest that the growth-inhibitory effects of n-3

PUFA on mammary tumor cells may be related more to the increased enrichment of n-3 PUFA in membrane phospholipids rather than to a decrease in membrane AA levels, at least in the MCF-7 cell line.

Interestingly, treatment with 60 μM EPA + 40 μM DHA increased the levels of saturated fatty acids, and decreased the concentration of polyunsaturated fatty acids, relative to the LA control treatments. This occurred in both cell lines and was significant particularly in PC and PS. This observation suggests that there is a compensation mechanism in place within the cells to try to maintain a certain level of membrane saturation, perhaps through increased fatty acid (i.e C16:0) synthesis.

These results also confirm that significant effects of n-3 PUFA on mammary tumor growth and death can be achieved with a lower level of incorporation of n-3 PUFA into membrane phospholipids (i.e. when EPA and DHA are provided in the presence of LA). The concentrations of n-3 PUFA in mammary tumor cell phospholipids following treatment with 45 μM EPA + 30 μM DHA + 75 μM LA observed in this study were consistent with n-3 PUFA concentrations in phospholipids of a rat mammary tumor (R3230AC) following administration of a diet containing 5 % w/w n-3 PUFA (Robinson *et al.*, 2002). For example, total n-3 PUFA concentrations in PE ranged from 9.9 to 23 % of the total fatty acids in MCF-7 and MDA-MB-231 cells, respectively, whereas total n-3 PUFA made up 14 % of the total fatty acids in PE of R3230AC mammary tumor cells. DHA itself comprised 1.8 to 8.7 % of the total fatty acids in PE in MCF-7 and MDA-MB-231 cells respectively, compared to 6.8 % of the total fatty acids in PE from the R3230AC mammary tumor *in vivo* (Robinson *et al.*, 2002). Thus the enrichment levels of n-3 PUFA in mammary tumor cells incubated with EPA and DHA plus LA *in vitro* observed in this thesis research are consistent with those that are attainable through dietary manipulation, supporting the relevance of this research to a dietary model of mammary tumor growth inhibition.

Our results also suggest an effect of EPA and DHA on cell survival signaling pathways. We demonstrated that EPA and DHA decreased the phosphorylation state (Ser473) of the Akt kinase. To our knowledge, this is the first study to show a decrease in Akt phosphorylation (Ser473) in tumor cells with n-3 PUFA treatment. Consistent with our data, a study by DeGraffenreid *et al.* (2003) has shown a decrease in Akt kinase activity with EPA treatment in transfected MCF-7 cells that overexpress constitutively active Akt. Since Akt requires translocation to the plasma membrane for activation, it is possible that tumor cell membrane enrichment of n-3 fatty acids after exposure to n-3 PUFA, as observed in the present study, might affect the activation of signaling molecules such as Akt that are recruited to the membrane for activation.

Additionally, we observed that n-3 PUFA reduced the activity, or DNA-binding capacity, of NFκB in MDA-MB-231 cells, possibly as a result of decreased Akt phosphorylation. A number of studies in monocytes/macrophages have demonstrated the ability of n-3 PUFA to decrease NFκB activity *in vitro* (Lee *et al.*, 2003; Novak *et al.*, 2003; Lee *et al.*, 2004; Zhao *et al.*, 2004). Cells treated with 45 μM EPA + 30 μM DHA + 75 μM LA had somewhat higher levels of NFκB DNA binding activity than cells treated with 60 μM EPA + 40 μM DHA. This may have resulted from a stimulatory influence of LA on NFκB DNA binding activity, as LA has been shown to increase NFκB activity in other cell types (Hennig *et al.*, 1996; Hennig *et al.*, 2000). Nevertheless, since Akt and NFκB play a role in promoting cell survival and inhibiting apoptosis, and since inhibition of NFκB activity has been shown to render tumor cells susceptible to apoptosis (Sovak *et al.*, 1997), the decreases in Akt phosphorylation and NFκB activity induced by n-3 PUFA observed in this study suggest a novel mechanism by which n-3 PUFA decrease the growth and survival of breast cancer cells, at least in MDA-MB-231 cells.

We could not detect an effect of n-3 PUFA on NFκB DNA-binding activity in MCF-7 cells. Consistent with their ER-positive status and hormone-dependent growth, MCF-7 cells have very low basal levels of NFκB DNA-binding activity (Nakshatri *et al.*, 1997; Sliva *et al.*, 2002; Pratt *et al.*, 2003). Thus it is possible that in this study, n-3 PUFA treatment could not perceptibly decrease an already low level of NFκB activity. In fact, other researchers have reported difficulty measuring NFκB activity in this cell line due to insufficient NFκB DNA-binding activity under normal cell culture conditions (Pratt *et al.*, 2003). NFκB activity could only be characterized in MCF-7 cells grown in estradiol-free medium (in which NFκB activity is induced) (Pratt *et al.*, 2003). Consistent with the failure to detect NFκB activity in MCF-7 cells, this cell line was much less sensitive to pharmacological inhibition of NFκB than was the MDA-MB-231 cell line, which exhibits high constitutive NFκB DNA-binding activity (Nakshatri *et al.*, 1997; Sliva *et al.*, 2002). In the present experiment, MDA-MB-231 cells, seeded at twice the cell density as MCF-7 cells, experienced similar reductions in cell growth and viability to MCF-7 cells only when treated with a 10-fold lower dose of MG-132 as compared to MCF-7 cells. Pratt *et al.* suggest that hormones such as estrogen provide the primary growth and survival stimulus for early stage, hormone-dependent breast tumor cells. However, during tumor progression from hormone-dependent to independent growth, breast tumor cells may come to rely less on estrogen for growth stimulation and more on their intrinsic NFκB activity, which increases as cells progress toward hormone-independence (Pratt *et al.*, 2003). In their proposed model, growth stimulation by estrogen may become less important for tumor cell growth, as increasing NFκB activity becomes the primary signal for cell growth and survival. Under such a model, inhibition of NFκB activity in hormone-independent cells with high constitutive NFκB activity is expected to have more severe consequences than would NFκB inhibition in hormone-dependent cells, whose primary growth stimulatory pathway involves estrogen (Pratt *et al.*, 2003; and Dr. M.A.C. Pratt, personal communication). The results of this study, showing greater reductions in cell growth and viability in the MDA-MB-231 cell line versus the MCF-7 cell line with MG-132 treatment, are in accordance with such a model. Nevertheless, the fact that decreases in NFκB DNA-binding activity with n-3 PUFA treatment could not be detected in MCF-7 cells, despite significant growth inhibition of these cells by n-3 PUFA, raises the possibility that the mechanism by which n-3 PUFA inhibit breast cancer cell growth involves pathways other than NFκB, at least in MCF-7 (or ER-positive early stage) cells.

In conclusion, the current study supports the hypothesis that n-3 PUFA decrease proliferation and increase apoptosis of human breast cancer cells, potentially through inhibition of signaling through the Akt/NFκB cell survival signaling pathway. Consistent with these effects we observed incorporation of EPA and DHA into the major membrane phospholipids. Understanding the mechanism(s) by which n-3 PUFA alter the growth or death of breast cancer cells is a necessary step in determining how these fatty acids may interact with current anti-cancer therapies and thus the clinical potential of these dietary fats in the treatment of breast cancer.

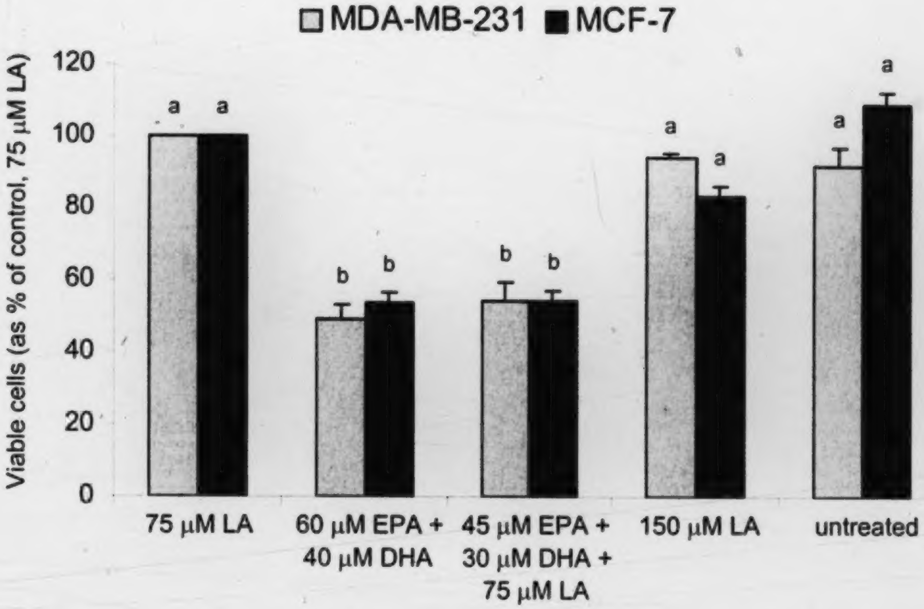

Figure 3-1 Effect of fatty acids on MDA-MB-231 and MCF-7 breast cancer cell growth. Viable cells were counted to determine cell population growth after 72 hours exposure to fatty acids, as described in "Materials and Methods". Cells cultured in parallel but not exposed to exogenous fatty acids (untreated cells) are shown for comparison. Within each cell line, values were normalized to a percentage of the control treatment (75 μM LA), which was taken as 100 %. Bars represent the means ± SE of 17 to 21 experiments. Within each cell line, bars that do not share a letter are significantly different ($p<0.05$).

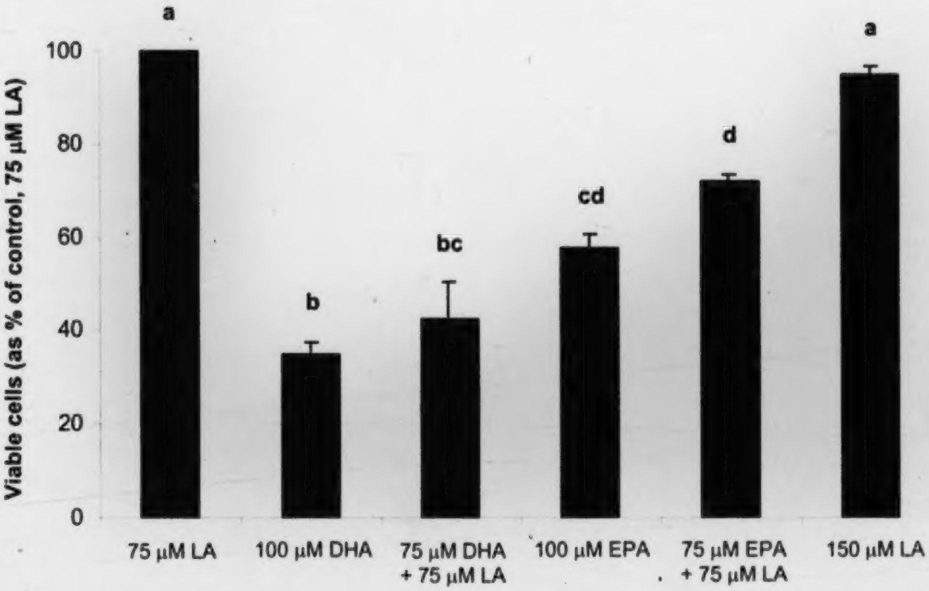

Figure 3-2 Effect of EPA versus DHA on the growth of MDA-MB-231 cells. Viable cells were counted to determine cell population growth after 72 hours exposure to fatty acids, as described in "Materials and Methods". All values were normalized to a percentage of the control treatment (75 µM LA), which was taken as 100 %. Bars represent the means ± SE of 6 experiments. Bars that do not share a letter are significantly different ($p<0.05$).

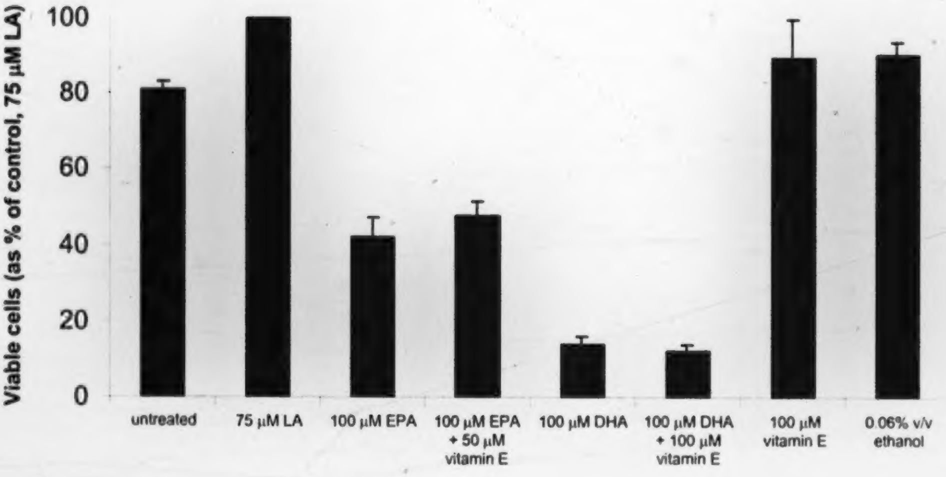

Figure 3-3 Effect of vitamin E on n-3 PUFA-induced growth inhibition of MDA-MB-231 cells. Viable cells were counted to determine cell population growth after 72 hours exposure to EPA or DHA ± vitamin E, as described in "Materials and Methods". All values were normalized to a percentage of the control treatment (75 µM LA), which was taken as 100 %. Bars represent the means ± SD of 2 to 3 experiments.

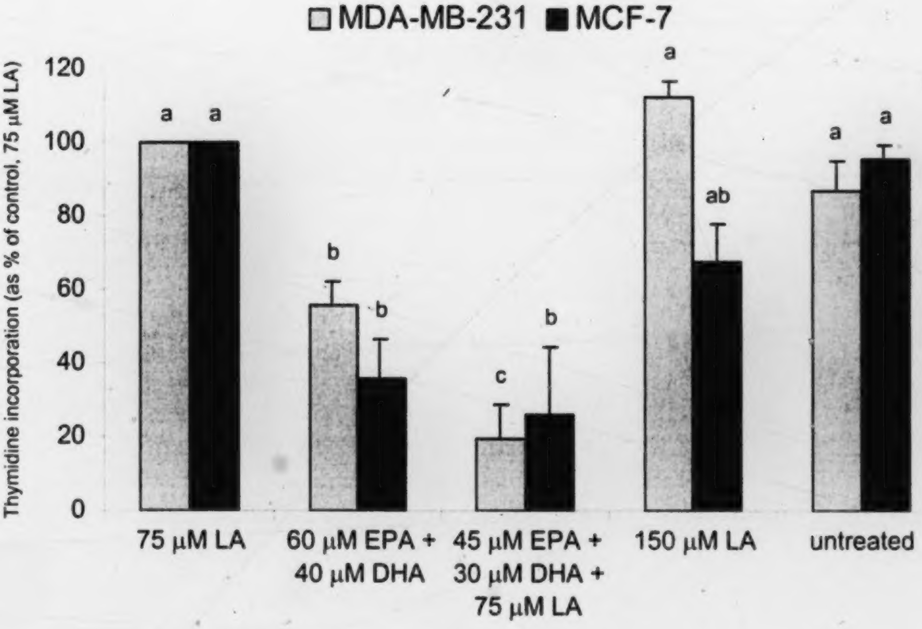

Figure 3-4 Effect of n-3 PUFA on [methyl-^{3}H]-thymidine incorporation by MDA-MB-231 and MCF-7 cells. Cells were cultured with fatty acids for 72 hours and [methyl-^{3}H]-thymidine incorporation was measured as described in "Materials and Methods". Disintegrations per minute (DPM) were corrected for final cell number in each fatty acid treatment using the corresponding cell count data from parallel growth experiments. Cells cultured in parallel but not exposed to exogenous fatty acids (untreated cells) are shown for comparison. Within each cell line, the corrected DPMs are expressed as a percentage of the control treatment (75 μM LA), which was taken as 100 %. Bars represent the means ± SE of 4 to 5 experiments. Within each cell line, bars that do not share a letter are significantly different ($p<0.05$).

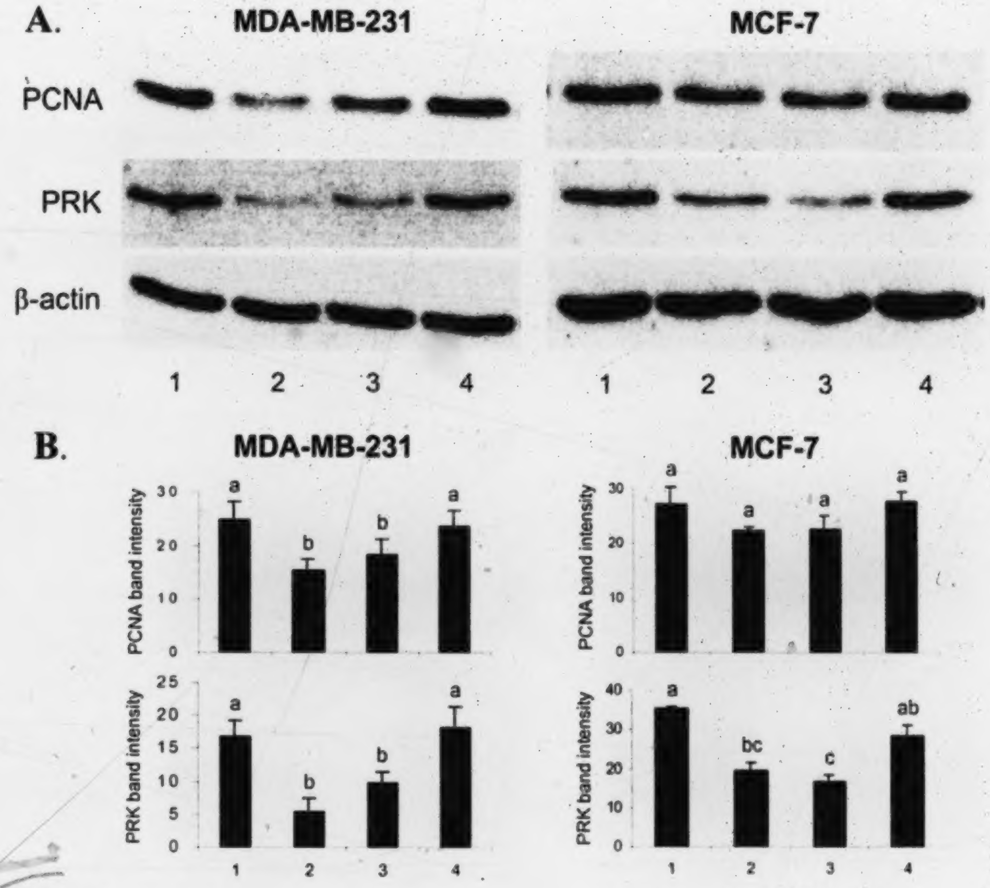

Figure 3-5 Effect of n-3 PUFA on PCNA and PRK expression in breast cancer cells, analyzed by western blotting after 72 hours of fatty acid exposure. **A**, Results shown are representative of 4-6 (MDA-MB-231) and 3 (MCF-7) independent experiments. β-actin expression confirms equal protein loading. **B**, The histograms depict PCNA and PRK band intensities (means ± SE) as determined by densitometric scanning of western blot autoradiograms. Bars that do not share a letter are significantly different ($p<0.05$). 1 = 75 μM LA; 2 = 60 μM EPA + 40 μM DHA; 3 = 45 μM EPA + 30 μM DHA + 75 μM LA; 4 = 150 μM LA.

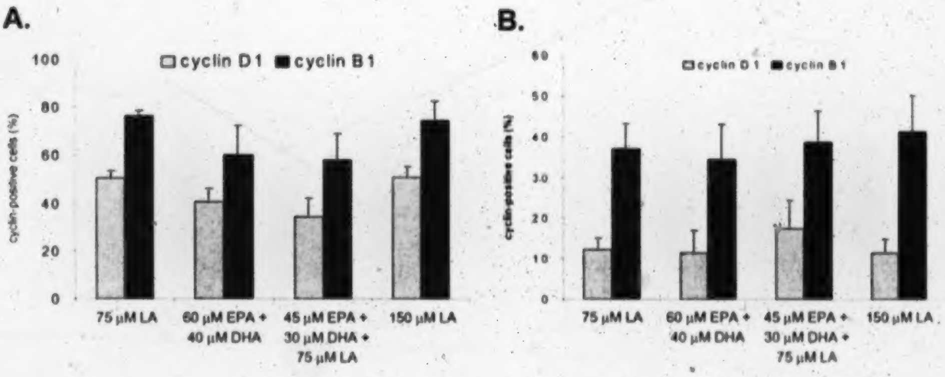

Figure 3-6 Effect of n-3 PUFA on cyclin protein expression in MDA-MB-231 (**A**) and MCF-7 (**B**) cells. The percentage of cyclin D1- and cyclin B1-positive cells following 72 hours exposure to fatty acids was determined as described in "Materials and Methods". Bars represent the means ± SE of 3 experiments. There were no significant differences in the percentages of cyclin D1- or cyclin B1-positive cells among fatty acid treatments in either cell line.

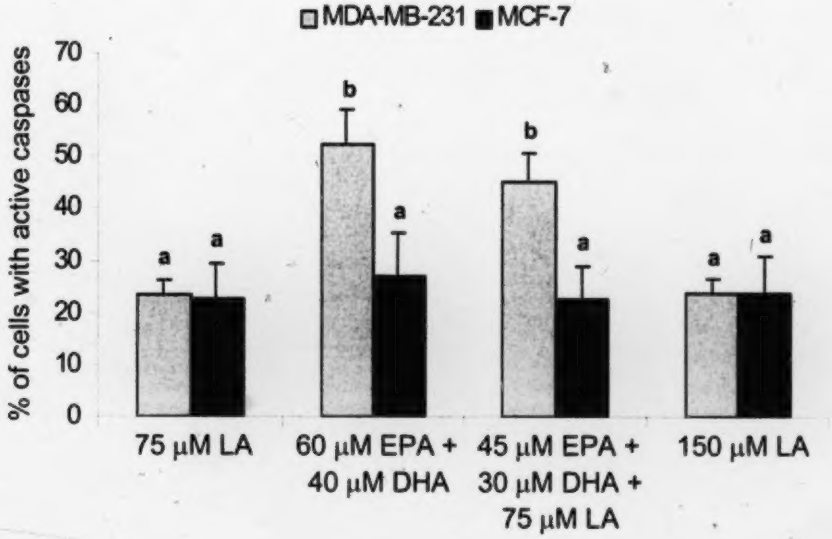

Figure 3-7 Effect of n-3 PUFA on caspase protein activity in breast cancer cells following exposure to fatty acids for 72 hours. The percentage of cells with activated caspases was determined as described in "Materials and Methods". Bars represent the means ± SE of 5 to 6 experiments. Within each cell line, bars that do not share a letter are significantly different ($p<0.05$).

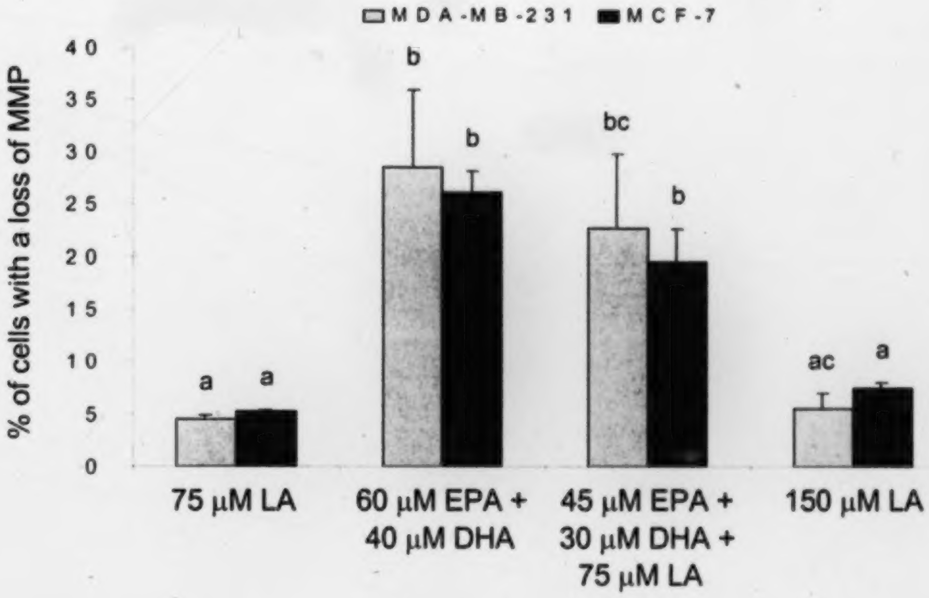

Figure 3-8 Effect of n-3 PUFA on mitochondrial membrane potential in breast cancer cells after 72 hours exposure to fatty acids. The percentage of cells with a loss of mitochondrial membrane potential was determined as described in "Materials and Methods". Bars represent the means ± SE of 4 to 5 experiments. Within each cell line, bars that do not share a letter are significantly different ($p<0.05$).

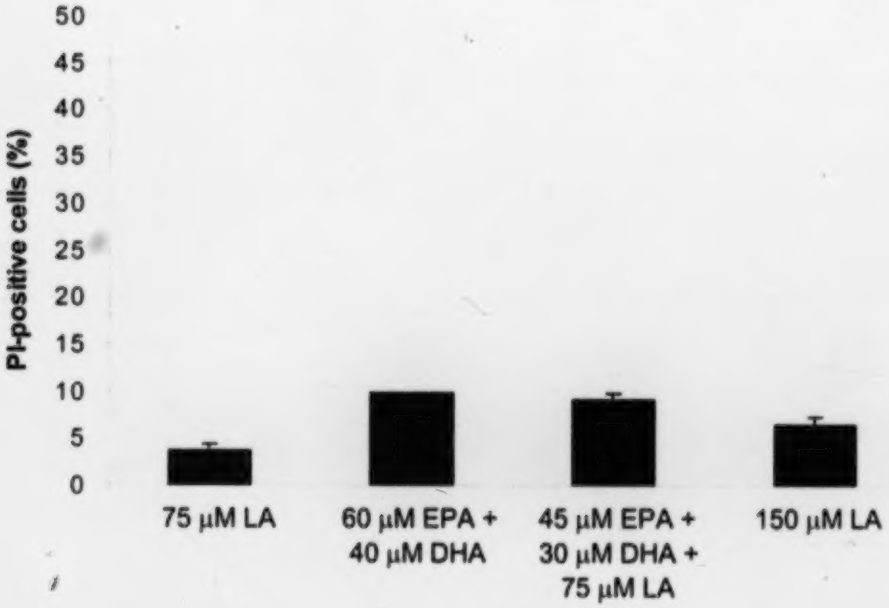

Figure 3-9 Effect of n-3 PUFA on propidium iodide (PI) uptake by MDA-MB-231 cells. The percentage of PI-positive cells following exposure to fatty acids for 72 hours was determined as described in "Materials and Methods". Bars represent the means ± SD of 1 to 2 experiments.

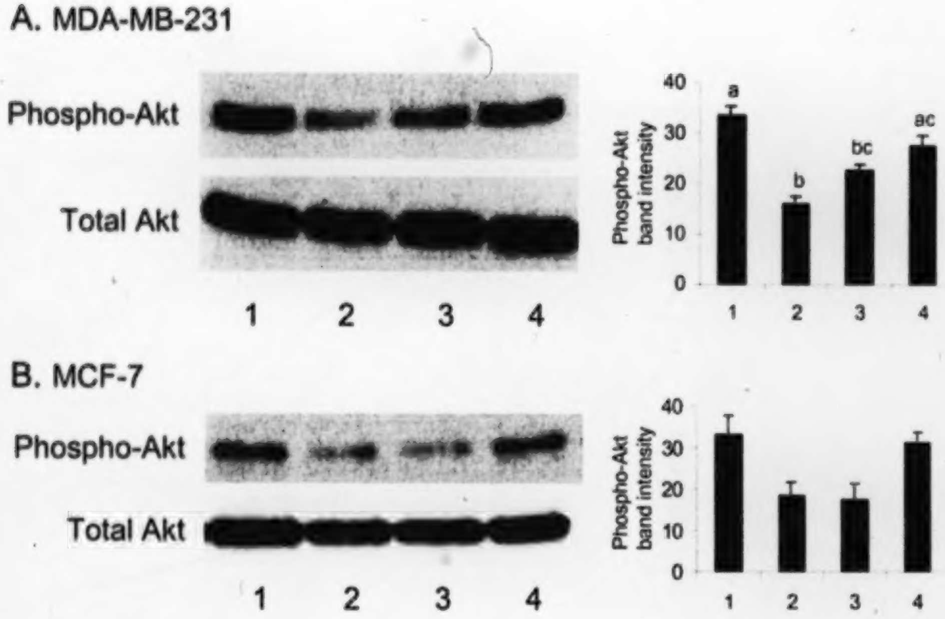

Figure 3-10 Effect of n-3 PUFA on phosphorylated (Ser473) Akt and total Akt protein levels in breast cancer cells, analyzed by western blotting after 72 hours of fatty acid exposure. **A**, Results shown are representative of 5 independent experiments with MDA-MB-231 cells. The histogram depicts phospho-Akt band intensities (means ± SE) as determined by densitometric scanning of western blot autoradiograms. Bars that do not share a letter are significantly different ($p<0.05$). **B**, Results shown are representative of 1-2 independent experiments with MCF-7 cells. The histogram depicts phospho-Akt band intensities (means ± SD) as determined by densitometric scanning of western blot autoradiograms. 1 = 75 μM LA; 2 = 60 μM EPA + 40 μM DHA; 3 = 45 μM EPA + 30 μM DHA + 75 μM LA; 4 = 150 μM LA.

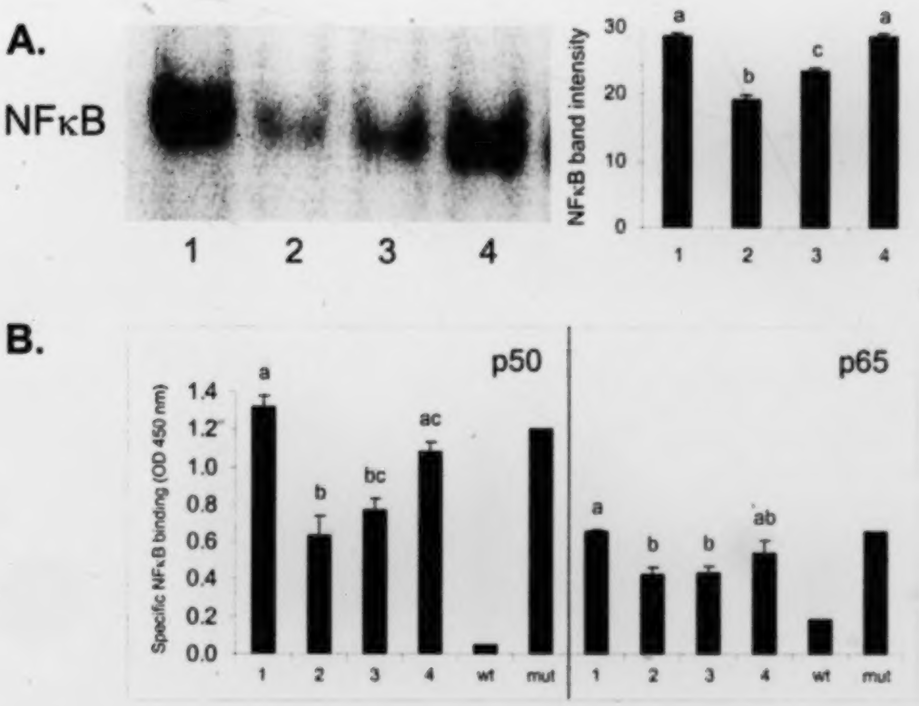

Figure 3-11 Effect of n-3 PUFA on NFκB DNA binding activity in MDA-MB-231 cells after 72 hours exposure to fatty acids. **A**, Nuclear extracts (5 μg) were analyzed by electrophoretic mobility shift assay (EMSA) for DNA binding activity of NFκB, as described in "Materials and Methods". The results shown are representative of 3 independent experiments. The histogram depicts NFκB band intensities (means ± SE) as determined by densitometric scanning of EMSA autoradiograms. Bars that do not share a letter are significantly different ($p<0.05$). **B**, DNA binding of active NFκB subunits (p50 and p65) was confirmed using the TransAM™ NFκB ELISA Kit as described in "Materials and Methods". To confirm specific binding of NFκB subunits, nuclear lysates (from LA-treated cells) were assayed in the presence of soluble wild-type (wt) and mutant (mut) consensus oligonucleotides. The results shown are the means ± SE of 3 independent experiments. Bars that do not share a letter are significantly different ($p<0.05$). 1 = 75 μM LA; 2 = 60 μM EPA + 40 μM DHA; 3 = 45 μM EPA + 30 μM DHA + 75 μM LA; 4 = 150 μM LA.

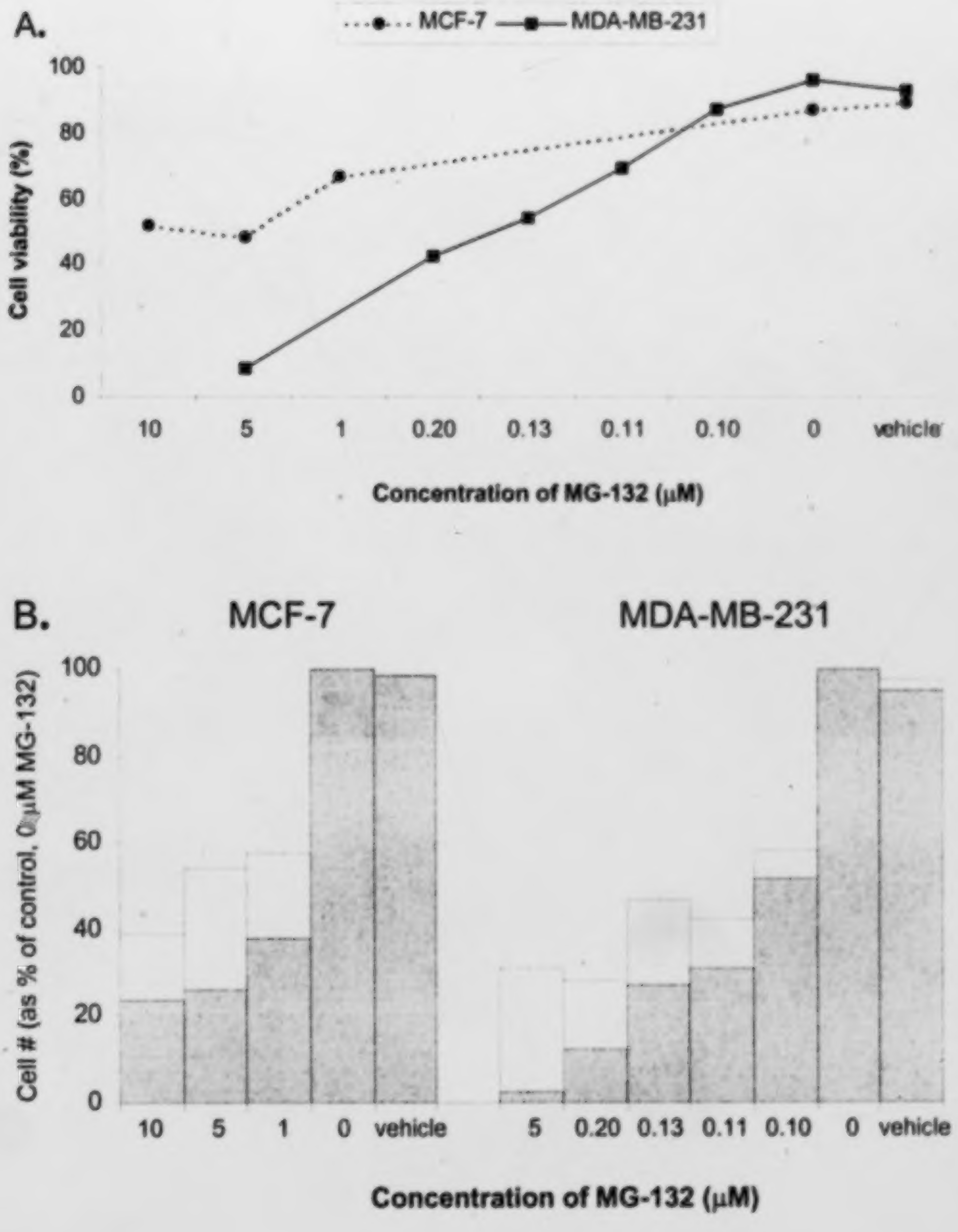

Figure 3-12 Effect of MG-132 on human breast cancer cell growth and viability. Viable and non-viable cells were counted to determine cell viability (**A**) and cell population growth (**B**) after 48 hours exposure to MG-132 or vehicle (0.1 % v/v DMSO) treatment,

as described in "Materials and Methods". **A**, Cell viability was calculated as [number of viable cells/(number of viable + non-viable cells) x 100]. **B**, The total number of cells as well as the number of viable cells (shaded portion) were normalized to a percentage of total number and number of viable cells, respectively, in the control treatment (0 μM MG-132). Values are representative of 1 to 2 experiments.

Table 3-1 Effect of n-3 PUFA on induction of apoptosis in breast cancer cells

	Absorbance (OD value)†	
Treatment	**MDA-MB-231**	**MCF-7**
75 μM LA	0.06 ± 0.02^{a}	0.09 ± 0.03^{a}
60 μM EPA + 40 μM DHA	1.48 ± 0.44^{b}	0.91 ± 0.33^{b}
45 μM EPA + 30 μM DHA + 75 μM LA	0.55 ± 0.28^{bc}	0.73 ± 0.13^{b}
150 μM LA	0.09 ± 0.02^{a}	0.14 ± 0.02^{a}
untreated cells	0.11 ± 0.04^{ac}	0.06 ± 0.01^{a}

† Absorbance values (reflecting the extent of DNA fragmentation in cytoplasmic cell lysates) are the mean ± SE of 3 separate experiments. Absorbance values were log-transformed prior to statistical analysis. Values within a column that do not share a letter are significantly different ($p<0.05$).

Table 3-2 Effect of n-3 PUFA on lactate dehydrogenase release from breast cancer cells

	MDA-MB-231		MCF-7	
Fatty acid treatment	**Absorbance**[†]	**% of control**	**Absorbance**	**% of control**
75 μM LA	0.233 ± 0.067	-	0.106 ± 0.038	-
60 μM EPA + 40 μM DHA	0.257 ± 0.069	110	0.107 ± 0.036	101
45 μM EPA + 30 μM DHA + 75 μM LA	0.258 ± 0.097	111	0.095 ± 0.054	90
150 μM LA	0.238 ± 0.082	102	0.095 ± 0.040	90

† Absorbance (OD) values represent the means ± SE of 4 (MCF-7) and 5 (MDA-MB-231) experiments.
There were no significant differences in LDH release among fatty acid treatments in either cell line.

Table 3-3 Fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol of MDA-MB-231 breast cancer cells following treatment with polyunsaturated fatty acids[1]

	Phosphatidylcholine				Phosphatidylethanolamine				Phosphatidylinositol			
	75 μM LA	60 μM EPA + 40 μM DHA +	45 μM EPA + 30 μM DHA + 75 μM LA	150 μM LA	75 μM LA	60 μM EPA + 40 μM DHA +	45 μM EPA + 30 μM DHA + 75 μM LA	150 μM LA	75 μM LA	60 μM EPA + 40 μM DHA +	45 μM EPA + 30 μM DHA + 75 μM LA	150 μM LA
Fatty acid		**% w/w of total fatty acids**				**% w/w of total fatty acids**				**% w/w of total fatty acids**		
C14:0	1.13 ± 0.1^{a}	1.79 ± 0.2^{b}	1.51 ± 0.2^{ab}	1.01 ± 0.1^{a}	0.25 ± 0.1	0.32 ± 0.1	0.20 ± 0.0	0.24 ± 0.1	0.08 ± 0.0	0.05 ± 0.0	0.15 ± 0.0	0.14 ± 0.0
C15:0	0.25 ± 0.0^{a}	0.38 ± 0.0^{b}	0.34 ± 0.0^{b}	0.24 ± 0.0^{a}	0.07 ± 0.0	0.07 ± 0.0	0.08 ± 0.0	0.10 ± 0.0	ND	0.03 ± 0.0	0.10 ± 0.0	0.07 ± 0.0
C16:0	21.44 ± 0.3^{a}	33.19 ± 1.1^{b}	26.79 ± 0.9^{c}	20.03 ± 0.9^{a}	6.11 ± 1.0^{a}	9.55 ± 1.1^{b}	6.86 ± 0.7^{a}	5.87 ± 0.3^{a}	2.14 ± 0.5^{a}	3.36 ± 0.4^{b}	3.13 ± 0.5^{b}	3.01 ± 0.7^{ab}
C16:1 (n-7)	0.71 ± 0.1^{a}	1.21 ± 0.0^{b}	1.08 ± 0.1^{b}	0.67 ± 0.1^{a}	0.28 ± 0.0^{a}	0.30 ± 0.0^{a}	0.29 ± 0.0^{a}	0.19 ± 0.0^{b}	0.07 ± 0.0	0.08 ± 0.0	0.10 ± 0.0	0.07 ± 0.0
C17:0	0.51 ± 0.0^{a}	0.56 ± 0.0^{b}	0.56 ± 0.0^{b}	0.48 ± 0.0^{a}	0.52 ± 0.1^{a}	0.77 ± 0.1^{b}	0.63 ± 0.1^{c}	0.55 ± 0.1^{a}	0.39 ± 0.1^{a}	0.71 ± 0.1^{b}	0.62 ± 0.1^{b}	0.51 ± 0.1^{c}
C17:1 (n-7)	0.70 ± 0.1^{a}	0.77 ± 0.0^{a}	0.71 ± 0.1^{a}	0.56 ± 0.0^{b}	6.60 ± 1.3^{a}	4.24 ± 0.4^{b}	5.47 ± 0.7^{ab}	5.30 ± 1.0^{ab}	0.13 ± 0.0	0.13 ± 0.0	0.14 ± 0.0	0.13 ± 0.0
C18:0	8.93 ± 0.5^{a}	8.66 ± 0.8^{a}	6.03 ± 0.9^{b}	6.12 ± 0.8^{b}	15.35 ± 1.5^{a}	25.69 ± 0.2^{b}	22.32 ± 0.5^{ab}	17.26 ± 1.8^{ab}	41.02 ± 0.3^{a}	43.57 ± 0.5^{b}	42.29 ± 1.3^{ab}	41.22 ± 0.4^{ab}
C18:1 (n-7 + n-9)	9.86 ± 0.3^{a}	11.93 ± 0.8^{b}	8.90 ± 0.6^{a}	7.47 ± 0.4^{c}	11.70 ± 0.7^{a}	9.14 ± 0.3^{b}	9.97 ± 0.4^{b}	9.72 ± 0.3^{b}	4.85 ± 0.4^{ab}	5.03 ± 0.1^{a}	4.32 ± 0.4^{ab}	3.92 ± 0.6^{b}
C18:2 (n-6)	**34.74 ± 2.0^{a}**	**1.68 ± 0.1^{b}**	**39.18 ± 1.6^{ac}**	**43.06 ± 1.4^{c}**	**22.85 ± 3.3^{a}**	**0.78 ± 0.0^{b}**	**20.89 ± 1.6^{a}**	**26.10 ± 2.6^{a}**	**14.20 ± 3.4^{a}**	**1.06 ± 0.1^{b}**	**20.17 ± 3.0^{a}**	**20.75 ± 3.3^{a}**
C18:3 (n-3)	0.03 ± 0.0^{a}	0.18 ± 0.0^{b}	0.09 ± 0.0^{a}	0.03 ± 0.0^{a}	0.16 ± 0.0	0.18 ± 0.0	0.17 ± 0.0	0.16 ± 0.0	ND	0.05 ± 0.0	0.02 ± 0.0	0.01 ± 0.0
C20:0	0.13 ± 0.0^{a}	0.14 ± 0.0^{a}	0.11 ± 0.0^{b}	0.09 ± 0.0^{b}	0.17 ± 0.0^{a}	0.26 ± 0.0^{b}	0.18 ± 0.0^{a}	0.16 ± 0.0^{a}	0.24 ± 0.0	0.77 ± 0.2	0.35 ± 0.1	0.51 ± 0.3
C20:1 (n-7 + n-9)	0.40 ± 0.0^{a}	0.19 ± 0.0^{b}	0.16 ± 0.0^{b}	0.27 ± 0.0^{c}	0.42 ± 0.0^{a}	0.21 ± 0.0^{b}	0.21 ± 0.0^{b}	0.35 ± 0.0^{a}	0.15 ± 0.0^{a}	0.07 ± 0.0^{b}	0.07 ± 0.0^{b}	0.11 ± 0.0^{ab}
C20:2 (n-6)	8.47 ± 0.5^{a}	0.16 ± 0.0^{b}	3.82 ± 0.2^{c}	11.21 ± 0.8^{d}	6.33 ± 1.0^{a}	0.16 ± 0.0^{b}	3.32 ± 0.2^{c}	9.04 ± 0.9^{a}	2.91 ± 0.3^{ac}	0.41 ± 0.1^{b}	1.71 ± 0.3^{a}	3.81 ± 0.6^{c}
C20:3 (n-6)	1.82 ± 0.3^{a}	0.59 ± 0.0^{b}	0.58 ± 0.1^{b}	1.34 ± 0.1^{a}	2.09 ± 0.0^{a}	0.55 ± 0.0^{b}	0.79 ± 0.1^{b}	1.78 ± 0.1^{a}	3.95 ± 0.6^{a}	1.31 ± 0.1^{b}	3.09 ± 0.5^{ab}	3.89 ± 0.6^{a}
C20:4 (n-6)	**3.93 ± 0.7^{a}**	**2.34 ± 0.0^{b}**	**1.14 ± 0.1^{c}**	**2.50 ± 0.5^{b}**	**12.90 ± 2.1^{a}**	**3.80 ± 0.3^{b}**	**4.17 ± 0.6^{b}**	**10.16 ± 1.6^{a}**	**24.90 ± 4.6^{a}**	**9.86 ± 1.6^{b}**	**11.12 ± 2.7^{bc}**	**18.38 ± 4.6^{ac}**
C20:5 (n-3)	**0.09 ± 0.0^{a}**	**12.99 ± 1.3^{b}**	**2.11 ± 0.1^{c}**	**0.06 ± 0.0^{a}**	**0.20 ± 0.1^{a}**	**14.08 ± 0.5^{b}**	**6.03 ± 0.4^{c}**	**0.15 ± 0.0^{a}**	**0.14 ± 0.0^{a}**	**15.78 ± 0.9^{b}**	**4.51 ± 0.1^{c}**	**0.08 ± 0.0^{a}**
C22:0	0.32 ± 0.0	0.19 ± 0.1	0.19 ± 0.0	0.30 ± 0.0	0.19 ± 0.0	0.15 ± 0.0	0.18 ± 0.0	0.27 ± 0.1	0.20 ± 0.0^{ab}	0.36 ± 0.1^{a}	0.27 ± 0.1^{ab}	0.17 ± 0.1^{b}
C22:2 (n-6)	1.02 ± 0.1^{a}	0.04 ± 0.0^{b}	0.35 ± 0.0^{c}	1.29 ± 0.1^{d}	0.58 ± 0.1^{a}	0.06 ± 0.0^{b}	0.24 ± 0.0^{b}	0.98 ± 0.1^{c}	0.17 ± 0.0	0.08 ± 0.1	0.21 ± 0.1	0.25 ± 0.1
C22:4 (n-6)	2.36 ± 0.4^{a}	0.54 ± 0.0^{bc}	0.27 ± 0.0^{c}	1.36 ± 0.2^{b}	7.00 ± 0.9^{a}	0.71 ± 0.0^{b}	0.84 ± 0.0^{b}	6.27 ± 0.6^{a}	2.49 ± 0.1^{a}	0.53 ± 0.0^{b}	0.50 ± 0.0^{b}	1.57 ± 0.3^{c}
C22:5 (n-3)	**0.74 ± 0.1^{a}**	**13.45 ± 0.5^{b}**	**3.39 ± 0.2^{c}**	**0.42 ± 0.1^{d}**	**2.29 ± 0.2^{a}**	**13.10 ± 0.7^{b}**	**8.05 ± 0.4^{c}**	**1.96 ± 0.2^{a}**	**0.65 ± 0.1^{a}**	**10.29 ± 0.4^{b}**	**4.22 ± 0.3^{c}**	**0.40 ± 0.0^{a}**
C24:0	0.55 ± 0.1^{a}	0.18 ± 0.1^{b}	0.21 ± 0.0^{b}	0.27 ± 0.1^{b}	0.15 ± 0.0^{a}	0.06 ± 0.0^{b}	0.12 ± 0.0^{ab}	0.18 ± 0.0^{a}	0.24 ± 0.1	0.22 ± 0.1	0.26 ± 0.1	0.20 ± 0.0
C22:6 (n-3)	**0.68 ± 0.1^{a}**	**8.51 ± 0.4^{b}**	**2.07 ± 0.2^{c}**	**0.44 ± 0.1^{a}**	**3.31 ± 0.3^{a}**	**15.54 ± 0.8^{b}**	**8.67 ± 0.7^{c}**	**2.89 ± 0.2^{a}**	**0.42 ± 0.1^{a}**	**5.86 ± 0.3^{b}**	**2.03 ± 0.1^{c}**	**0.29 ± 0.1^{a}**
C24:1 (n-9)	0.48 ± 0.0^{a}	0.01 ± 0.0^{b}	0.08 ± 0.1^{b}	0.25 ± 0.0^{c}	0.02 ± 0.0	ND	ND	0.01 ± 0.0	0.16 ± 0.1	0.09 ± 0.0	0.16 ± 0.1	0.12 ± 0.0
Σ n-3 PUFA	1.57 ± 0.3^{a}	35.16 ± 2.1^{b}	7.69 ± 0.4^{c}	0.98 ± 0.1^{a}	5.98 ± 0.6^{a}	42.94 ± 0.5^{b}	22.96 ± 1.5^{c}	5.20 ± 0.5^{a}	1.22 ± 0.2^{a}	31.98 ± 1.5^{b}	10.77 ± 0.3^{c}	0.78 ± 0.1^{a}
Σ n-6 PUFA	42.85 ± 0.9^{a}	5.23 ± 0.1^{b}	41.18 ± 1.5^{a}	48.27 ± 0.8^{c}	45.06 ± 2.3^{a}	5.86 ± 0.4^{b}	26.76 ± 0.9^{c}	44.31 ± 0.5^{a}	45.74 ± 1.2^{a}	12.79 ± 1.6^{b}	35.00 ± 0.4^{c}	44.76 ± 1.4^{a}
Σ SFA	33.88 ± 0.8^{ac}	45.20 ± 1.6^{b}	35.95 ± 0.6^{a}	28.96 ± 1.5^{c}	22.90 ± 2.0^{a}	36.99 ± 1.3^{b}	30.65 ± 1.3^{ab}	24.75 ± 2.2^{a}	44.57 ± 0.4^{a}	49.31 ± 0.5^{b}	47.45 ± 0.6^{bc}	46.00 ± 0.3^{ac}
Σ MUFA	12.21 ± 0.4^{a}	14.22 ± 0.8^{b}	11.01 ± 0.6^{c}	9.30 ± 0.4^{d}	19.16 ± 1.5^{a}	13.98 ± 0.5^{b}	16.07 ± 1.0^{c}	15.71 ± 1.1^{bc}	12.21 ± 0.4	14.22 ± 0.8	4.86 ± 0.4	4.39 ± 0.7
Σ PUFA	53.92 ± 1.2^{a}	40.59 ± 2.0^{b}	53.04 ± 1.2^{a}	61.75 ± 1.8^{c}	57.94 ± 2.5^{ab}	49.03 ± 0.9^{b}	53.28 ± 0.4^{ab}	59.54 ± 1.7^{a}	53.92 ± 1.2^{a}	40.59 ± 2.0^{b}	47.69 ± 0.2^{ab}	49.61 ± 0.9^{a}

[1] Values are percentages of total fatty acids and are expressed as means ± SE (n=3 per treatment). Abbreviations used: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detectable. Fatty acid data that were not normally distributed were log-transformed prior to statistical analysis. Values within a row and within each phospholipid class without a common superscript are significantly different ($p<0.05$). Values within a row with no superscripts are not significantly different.

Table 3-3 continued Fatty acid composition of phosphatidylserine and sphingomyelin of MDA-MB-231 breast cancer cells following treatment with polyunsaturated fatty acids

	Phosphatidylserine				Sphingomyelin			
	75 µM LA	60 µM EPA + 40 µM DHA +	45 µM EPA + 30 µM DHA + 75 µM LA	150 µM LA	75 µM LA	60 µM EPA + 40 µM DHA +	45 µM EPA + 30 µM DHA + 75 µM LA	150 µM LA
Fatty acid		**% w/w of total fatty acids**				**% w/w of total fatty acids**		
C14:0	0.23 ± 0.1^{a}	0.60 ± 0.1^{b}	0.57 ± 0.3^{ab}	0.28 ± 0.1^{ab}	1.03 ± 0.3	2.91 ± 0.4	1.38 ± 0.5	1.50 ± 0.2
C15:0	0.13 ± 0.1	0.18 ± 0.1	0.31 ± 0.2	0.08 ± 0.0	0.34 ± 0.1	0.68 ± 0.1	1.49 ± 0.9	0.41 ± 0.1
C16:0	9.02 ± 1.5^{a}	21.88 ± 2.4^{b}	13.83 ± 3.1^{a}	10.74 ± 1.3^{a}	49.54 ± 1.5	52.42 ± 1.7	42.67 ± 7.3	49.50 ± 3.3
C16:1 (n-7)	0.21 ± 0.0^{a}	0.44 ± 0.0^{b}	0.27 ± 0.0^{ab}	0.12 ± 0.1^{a}	0.19 ± 0.1	0.34 ± 0.2	0.39 ± 0.2	0.28 ± 0.1
C17:0	0.98 ± 0.1^{a}	1.40 ± 0.1^{b}	1.05 ± 0.2^{ab}	1.08 ± 0.1^{ab}	2.29 ± 0.1	1.55 ± 0.4	2.01 ± 0.4	1.99 ± 0.1
C17:1 (n-7)	0.44 ± 0.1	0.70 ± 0.1	0.67 ± 0.2	1.00 ± 0.4	0.14 ± 0.1	0.23 ± 0.1	0.32 ± 0.1	0.19 ± 0.1
C18:0	41.29 ± 1.6^{a}	39.78 ± 2.4^{ab}	38.66 ± 2.7^{ab}	38.18 ± 1.7^{b}	8.63 ± 0.9	10.15 ± 1.2	10.24 ± 2.0	7.49 ± 1.5
C18:1 (n-7 + n-9)	11.74 ± 1.0^{ab}	13.27 ± 0.6^{b}	9.81 ± 1.0^{a}	9.50 ± 1.5^{a}	1.78 ± 0.4	2.90 ± 1.0	3.36 ± 0.5	2.17 ± 1.0
C18:2 (n-6)	**19.56 ± 1.5^{a}**	**1.33 ± 0.1^{b}**	**21.23 ± 0.9^{a}**	**23.43 ± 2.1^{a}**	**3.64 ± 1.1**	**0.76 ± 0.2**	**7.82 ± 1.7**	**6.60 ± 3.0**
C18:3 (n-3)	ND	ND	0.04 ± 0.0	0.04 ± 0.0	ND	0.04 ± 0.0	0.17 ± 0.2	ND
C20:0	0.37 ± 0.0	0.41 ± 0.0	0.39 ± 0.0	0.39 ± 0.1	1.01 ± 0.2	1.22 ± 0.3	1.14 ± 0.2	0.83 ± 0.2
C20:1 (n-7 + n-9)	0.21 ± 0.0	0.13 ± 0.0	0.11 ± 0.1	0.15 ± 0.1	0.02 ± 0.0	ND	0.15 ± 0.2	ND
C20:2 (n-6)	3.52 ± 0.2^{a}	0.17 ± 0.0^{b}	1.98 ± 0.1^{c}	5.39 ± 0.6^{d}	0.63 ± 0.2	0.08 ± 0.1	0.78 ± 0.2	1.06 ± 0.4
C20:3 (n-6)	2.41 ± 0.5^{a}	0.83 ± 0.1^{b}	0.94 ± 0.2^{b}	2.00 ± 0.4^{a}	0.10 ± 0.1	0.05 ± 0.1	0.18 ± 0.2	0.17 ± 0.1
C20:4 (n-6)	**4.32 ± 0.6^{a}**	**0.88 ± 0.1^{b}**	**0.99 ± 0.1^{b}**	**2.67 ± 0.6^{ab}**	**0.47 ± 0.2**	**0.25 ± 0.0**	**0.41 ± 0.1**	**0.50 ± 0.2**
C20:5 (n-3)	**0.13 ± 0.0^{a}**	**2.57 ± 0.3^{b}**	**1.10 ± 0.2^{c}**	**0.07 ± 0.0^{a}**	**0.19 ± 0.1**	**1.13 ± 0.1**	**1.14 ± 0.6**	**0.35 ± 0.2**
C22:0	0.44 ± 0.1^{a}	0.38 ± 0.1^{a}	0.74 ± 0.1^{b}	0.63 ± 0.1^{ab}	7.69 ± 2.1	10.15 ± 1.5	7.48 ± 0.8	6.14 ± 1.5
C22:2 (n-6)	0.42 ± 0.0	ND	0.06 ± 0.1	0.39 ± 0.2	1.42 ± 0.2	1.02 ± 0.5	1.38 ± 0.3	1.98 ± 0.4
C22:4 (n-6)	2.56 ± 0.5^{a}	0.43 ± 0.0^{b}	0.42 ± 0.0^{b}	2.11 ± 0.2^{a}	0.15 ± 0.2	0.01 ± 0.0	ND	0.20 ± 0.1
C22:5 (n-3)	**0.68 ± 0.1^{a}**	**9.08 ± 1.5^{b}**	**3.34 ± 0.3^{c}**	**0.58 ± 0.1^{a}**	**0.07 ± 0.1^{a}**	**0.98 ± 0.1^{b}**	**0.67 ± 0.2^{b}**	**0.05 ± 0.1^{a}**
C24:0	0.09 ± 0.0	ND	0.23 ± 0.2	0.18 ± 0.1	10.02 ± 2.8	6.56 ± 0.5	8.25 ± 1.8	7.77 ± 1.8
C22:6 (n-3)	**0.72 ± 0.1^{a}**	**5.34 ± 0.8^{b}**	**2.85 ± 0.3^{c}**	**0.64 ± 0.0^{a}**	**0.02 ± 0.0^{a}**	**0.61 ± 0.1^{b}**	**0.22 ± 0.1^{a}**	**ND**
C24:1 (n-9)	ND	ND	0.05 ± 0.1	0.07 ± 0.1	9.90 ± 0.5	5.74 ± 1.4	7.95 ± 1.1	10.64 ± 3.2
Σ n-3 PUFA	1.52 ± 0.2^{a}	16.99 ± 2.0^{b}	7.33 ± 0.5^{c}	1.33 ± 0.1^{a}	0.28 ± 0.2^{a}	2.81 ± 0.1^{b}	2.19 ± 0.8^{ab}	0.42 ± 0.2^{a}
Σ n-6 PUFA	29.09 ± 0.4^{a}	3.47 ± 0.3^{b}	23.65 ± 0.7^{c}	30.33 ± 1.6^{a}	4.38 ± 1.2	1.11 ± 0.3	8.52 ± 1.8	7.52 ± 3.5
Σ SFA	52.71 ± 0.2^{a}	64.83 ± 1.1^{b}	55.96 ± 1.1^{a}	51.66 ± 0.9^{a}	81.17 ± 2.7	85.70 ± 0.7	74.72 ± 2.9	75.66 ± 2.1
Σ MUFA	12.73 ± 1.0^{ab}	14.54 ± 0.7^{b}	11.02 ± 1.0^{a}	10.90 ± 1.3^{a}	12.13 ± 1.2	9.29 ± 0.8	12.41 ± 1.3	13.37 ± 2.2
Σ PUFA	34.56 ± 0.8^{a}	20.63 ± 1.7^{b}	33.03 ± 0.3^{a}	37.44 ± 2.0^{a}	6.70 ± 1.6	5.01 ± 0.3	12.87 ± 1.9	10.97 ± 3.4

Table 3-4 Fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol of MCF-7 breast cancer cells following treatment with polyunsaturated fatty acids[1]

	Phosphatidylcholine				Phosphatidylethanolamine				Phosphatidylinositol			
	75 µM LA	60 µM EPA + 40 µM DHA +	45 µM EPA + 30 µM DHA + 75 µM LA	150 µM LA	75 µM LA	60 µM EPA + 40 µM DHA +	45 µM EPA + 30 µM DHA + 75 µM LA	150 µM LA	75 µM LA	60 µM EPA + 40 µM DHA +	45 µM EPA + 30 µM DHA + 75 µM LA	150 µM LA
Fatty acid	% w/w of total fatty acids				% w/w of total fatty acids				% w/w of total fatty acids			
C14:0	1.89 ± 0.0^{a}	4.31 ± 0.1^{b}	2.54 ± 0.1^{c}	1.90 ± 0.0^{a}	0.18 ± 0.0^{a}	0.32 ± 0.0^{b}	0.31 ± 0.0^{b}	0.17 ± 0.0^{a}	0.25 ± 0.0	0.24 ± 0.0	0.28 ± 0.1	0.20 ± 0.1
C15:0	0.30 ± 0.0	0.49 ± 0.0	0.36 ± 0.0	0.47 ± 0.2	0.05 ± 0.0	0.07 ± 0.0	0.09 ± 0.0	0.06 ± 0.0	0.15 ± 0.0	0.16 ± 0.1	0.13 ± 0.1	0.11 ± 0.0
C16:0	28.25 ± 0.3^{a}	39.47 ± 0.3^{b}	30.65 ± 0.4^{c}	27.31 ± 0.7^{a}	8.28 ± 0.8^{a}	11.26 ± 0.2^{b}	9.99 ± 0.4^{ab}	8.21 ± 0.1^{a}	7.02 ± 0.7^{ab}	8.45 ± 0.4^{b}	6.24 ± 0.8^{ab}	4.79 ± 0.3^{a}
C16:1 (n-7)	1.11 ± 0.0^{a}	2.44 ± 0.0^{b}	1.49 ± 0.0^{c}	0.81 ± 0.1^{d}	0.56 ± 0.0^{a}	0.69 ± 0.0^{b}	0.59 ± 0.0^{a}	0.43 ± 0.0^{c}	0.39 ± 0.1	0.57 ± 0.0	0.39 ± 0.0	0.23 ± 0.0
C17:0	0.32 ± 0.0	0.35 ± 0.0	0.35 ± 0.0	0.30 ± 0.0	0.62 ± 0.0^{ac}	0.80 ± 0.0^{b}	0.69 ± 0.0^{a}	0.58 ± 0.0^{c}	0.50 ± 0.0^{a}	0.74 ± 0.0^{b}	0.65 ± 0.0^{b}	0.48 ± 0.0^{a}
C17:1 (n-7)	0.32 ± 0.0^{a}	0.53 ± 0.0^{b}	0.32 ± 0.0^{a}	0.21 ± 0.0^{c}	0.39 ± 0.1	0.40 ± 0.0	0.37 ± 0.0	0.35 ± 0.0	0.10 ± 0.0	0.12 ± 0.0	0.09 ± 0.0	0.09 ± 0.0
C18:0	5.14 ± 0.8	6.75 ± 0.2	6.00 ± 1.1	5.28 ± 1.1	24.37 ± 0.5^{a}	31.30 ± 0.3^{b}	27.26 ± 0.2^{c}	24.29 ± 0.4^{a}	41.87 ± 0.3	41.95 ± 0.1	41.80 ± 0.9	43.88 ± 0.2
C18:1 (n-7 + n-9)	9.92 ± 0.3^{a}	14.84 ± 0.5^{b}	8.10 ± 0.1^{ac}	6.53 ± 1.0^{c}	13.51 ± 0.5^{a}	8.47 ± 0.1^{b}	9.33 ± 0.1^{b}	9.33 ± 0.2^{b}	4.82 ± 0.5^{a}	5.06 ± 0.3^{a}	3.91 ± 0.2^{ab}	3.06 ± 0.1^{b}
C18:2 (n-6)	**41.85 ± 0.4^{a}**	**1.34 ± 0.1^{b}**	**42.39 ± 0.5^{a}**	**47.30 ± 1.4^{c}**	**39.91 ± 0.2^{a}**	**0.85 ± 0.0^{b}**	**37.13 ± 0.1^{c}**	**46.06 ± 0.4^{d}**	**29.34 ± 0.8^{a}**	**1.42 ± 0.3^{b}**	**30.63 ± 0.5^{a}**	**36.34 ± 0.4^{c}**
C18:3 (n-3)	0.04 ± 0.0^{a}	0.19 ± 0.0^{b}	0.09 ± 0.0^{c}	0.04 ± 0.0^{a}	0.05 ± 0.1^{a}	0.21 ± 0.0^{b}	0.14 ± 0.0^{ab}	0.16 ± 0.0^{ab}	ND	ND	0.01 ± 0.0	ND
C20:0	0.16 ± 0.0	0.14 ± 0.0	0.13 ± 0.0	0.13 ± 0.0	0.36 ± 0.0^{a}	0.32 ± 0.0^{ab}	0.29 ± 0.0^{b}	0.31 ± 0.0^{ab}	0.23 ± 0.0^{a}	0.39 ± 0.0^{b}	0.40 ± 0.0^{bc}	0.33 ± 0.1^{c}
C20:1 (n-7 + n-9)	0.44 ± 0.0^{a}	0.05 ± 0.0^{b}	0.12 ± 0.0^{c}	0.26 ± 0.0^{d}	0.43 ± 0.1^{a}	0.11 ± 0.0^{b}	0.15 ± 0.0^{bc}	0.31 ± 0.0^{ac}	0.09 ± 0.0^{a}	0.02 ± 0.0^{b}	0.04 ± 0.0^{bc}	0.06 ± 0.0^{ac}
C20:2 (n-6)	6.74 ± 0.1^{a}	0.03 ± 0.0^{b}	1.72 ± 0.1^{c}	6.97 ± 0.2^{a}	4.26 ± 0.5^{a}	0.07 ± 0.0^{b}	1.48 ± 0.0^{c}	5.25 ± 0.1^{a}	2.93 ± 0.1^{a}	0.21 ± 0.0^{b}	0.64 ± 0.1^{b}	3.19 ± 0.2^{a}
C20:3 (n-6)	0.12 ± 0.0^{a}	0.42 ± 0.0^{b}	0.14 ± 0.0^{a}	0.07 ± 0.0^{c}	0.25 ± 0.0^{a}	0.53 ± 0.0^{b}	0.25 ± 0.0^{a}	0.16 ± 0.0^{c}	0.77 ± 0.1^{a}	0.72 ± 0.0^{a}	0.53 ± 0.0^{b}	0.45 ± 0.0^{b}
C20:4 (n-6)	**0.95 ± 0.1^{a}**	**1.45 ± 0.0^{b}**	**0.45 ± 0.0^{c}**	**0.40 ± 0.0^{c}**	**3.01 ± 0.2^{a}**	**2.67 ± 0.1^{a}**	**1.37 ± 0.0^{b}**	**1.27 ± 0.1^{b}**	**7.33 ± 0.2^{a}**	**4.62 ± 0.1^{b}**	**2.82 ± 0.1^{c}**	**3.71 ± 0.2^{d}**
C20:5 (n-3)	**0.06 ± 0.0^{a}**	**16.87 ± 0.2^{b}**	**2.08 ± 0.1^{c}**	**0.06 ± 0.0^{a}**	**0.09 ± 0.1^{a}**	**26.03 ± 0.3^{b}**	**5.12 ± 0.1^{c}**	**0.11 ± 0.0^{a}**	**0.19 ± 0.0^{a}**	**19.50 ± 0.3^{b}**	**4.80 ± 0.2^{c}**	**0.19 ± 0.0^{a}**
C22:0	0.09 ± 0.0	0.10 ± 0.0	0.07 ± 0.0	0.08 ± 0.0	0.22 ± 0.1	0.16 ± 0.0	0.20 ± 0.0	0.30 ± 0.0	0.21 ± 0.0^{a}	0.19 ± 0.0^{ab}	0.17 ± 0.0^{b}	0.21 ± 0.0^{a}
C22:2 (n-6)	1.05 ± 0.0^{a}	0.02 ± 0.0^{b}	0.25 ± 0.0^{c}	1.04 ± 0.0^{a}	0.47 ± 0.1^{a}	0.03 ± 0.0^{b}	0.14 ± 0.0^{b}	0.58 ± 0.0^{a}	0.22 ± 0.1	0.07 ± 0.0	0.09 ± 0.1	0.18 ± 0.0
C22:4 (n-6)	0.24 ± 0.0^{a}	0.21 ± 0.1^{ab}	0.08 ± 0.0^{b}	0.14 ± 0.0^{ab}	0.62 ± 0.1^{a}	0.26 ± 0.0^{b}	0.23 ± 0.0^{b}	0.48 ± 0.0^{a}	0.90 ± 0.1	0.37 ± 0.0	0.39 ± 0.2	0.60 ± 0.1
C22:5 (n-3)	**0.25 ± 0.0^{a}**	**5.45 ± 0.2^{b}**	**1.34 ± 0.1^{c}**	**0.16 ± 0.0^{d}**	**0.97 ± 0.1^{a}**	**9.04 ± 0.3^{b}**	**2.74 ± 0.2^{c}**	**0.67 ± 0.0^{d}**	**0.94 ± 0.1^{a}**	**7.46 ± 0.3^{b}**	**2.54 ± 0.2^{c}**	**0.63 ± 0.0^{d}**
C24:0	0.12 ± 0.0	0.10 ± 0.0	0.08 ± 0.0	0.10 ± 0.0	0.19 ± 0.1	0.10 ± 0.0	0.15 ± 0.0	0.24 ± 0.0	0.14 ± 0.1	0.15 ± 0.0	0.15 ± 0.0	0.18 ± 0.0
C22:6 (n-3)	**0.34 ± 0.0^{a}**	**4.27 ± 0.1^{b}**	**1.13 ± 0.1^{c}**	**0.18 ± 0.0^{d}**	**0.79 ± 0.1^{a}**	**6.16 ± 0.1^{b}**	**1.83 ± 0.1^{c}**	**0.44 ± 0.0^{d}**	**1.37 ± 0.1^{a}**	**7.36 ± 0.4^{b}**	**3.05 ± 0.4^{c}**	**0.83 ± 0.1^{a}**
C24:1 (n-9)	0.10 ± 0.0^{a}	ND	0.02 ± 0.0^{b}	0.10 ± 0.0^{a}	0.05 ± 0.0^{ab}	ND	0.01 ± 0.0^{a}	0.08 ± 0.0^{b}	0.07 ± 0.1	ND	0.02 ± 0.0	0.11 ± 0.0
Σ n-3 PUFA	0.72 ± 0.0^{a}	26.81 ± 0.3^{b}	4.64 ± 0.1^{c}	0.47 ± 0.0^{d}	1.90 ± 0.2^{a}	41.48 ± 0.5^{b}	9.85 ± 0.2^{c}	1.37 ± 0.1^{d}	2.51 ± 0.1^{a}	34.33 ± 0.5^{b}	10.41 ± 0.8^{c}	1.65 ± 0.1^{d}
Σ n-6 PUFA	43.17 ± 0.4^{a}	3.47 ± 0.1^{b}	43.07 ± 0.6^{a}	47.91 ± 1.4^{c}	43.80 ± 0.1^{a}	4.34 ± 0.1^{b}	38.99 ± 0.1^{c}	47.96 ± 0.3^{d}	38.45 ± 0.8^{a}	7.30 ± 0.2^{b}	34.54 ± 0.4^{c}	41.22 ± 0.2^{d}
Σ SFA	36.30 ± 0.6^{a}	51.72 ± 0.2^{b}	40.22 ± 0.6^{c}	35.62 ± 1.1^{a}	34.31 ± 0.5^{a}	44.33 ± 0.6^{b}	38.99 ± 0.1^{c}	34.29 ± 0.4^{a}	50.42 ± 0.3	52.32 ± 0.6	49.87 ± 1.3	50.21 ± 0.2
Σ MUFA	12.01 ± 0.4^{a}	17.96 ± 0.5^{b}	10.09 ± 0.2^{ac}	7.98 ± 0.9^{c}	15.26 ± 0.5^{a}	9.76 ± 0.1^{b}	10.55 ± 0.1^{b}	10.55 ± 0.2^{b}	5.47 ± 0.6^{ab}	5.77 ± 0.4^{a}	4.46 ± 0.2^{ab}	3.56 ± 0.1^{b}
Σ PUFA	51.69 ± 0.3^{a}	30.32 ± 0.4^{b}	49.68 ± 0.5^{a}	56.40 ± 1.6^{c}	50.43 ± 0.8^{a}	45.91 ± 0.5^{b}	50.46 ± 0.0^{a}	55.16 ± 0.2^{c}	44.11 ± 0.9^{ab}	41.91 ± 0.5^{b}	45.67 ± 1.1^{a}	46.23 ± 0.2^{a}

[1] Values are percentages of total fatty acids and are expressed as means ± SE (n=3 per treatment). Abbreviations used: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detectable. Fatty acid data that were not normally distributed were log-transformed prior to statistical analysis. Values within a row and within each phospholipid class without a common superscript are significantly different ($p < 0.05$). Values within a row with no superscripts are not significantly different.

Table 3-4 continued Fatty acid composition of phosphatidylserine and sphingomyelin of MCF-7 breast cancer cells following treatment with polyunsaturated fatty acids

	Phosphatidylserine				**Sphingomyelin**			
	75 µM LA	**60 µM EPA + 40 µM DHA +**	**45 µM EPA + 30 µM DHA + 75 µM LA**	**150 µM LA**	**75 µM LA**	**60 µM EPA + 40 µM DHA**	**45 µM EPA + 30 µM DHA + 75 µM LA**	**150 µM LA**
Fatty acid	**% w/w of total fatty acids**				**% w/w of total fatty acids**			
C14:0	0.35 ± 0.1	0.47 ± 0.1	0.30 ± 0.0	0.22 ± 0.0	1.74 ± 0.1	1.36 ± 0.1	1.89 ± 0.5	1.54 ± 0.4
C15:0	0.16 ± 0.0	0.32 ± 0.1	0.23 ± 0.0	0.16 ± 0.0	0.65 ± 0.1	0.49 ± 0.0	0.75 ± 0.1	0.74 ± 0.2
C16:0	8.90 ± 1.7	10.38 ± 1.0	8.83 ± 0.3	7.53 ± 0.5	49.52 ± 1.1^{a}	50.36 ± 1.7^{a}	44.42 ± 0.5^{b}	48.73 ± 0.9^{a}
C16:1 (n-7)	0.65 ± 0.2	0.96 ± 0.1	0.58 ± 0.1	0.41 ± 0.1	0.20 ± 0.2	0.07 ± 0.1	0.22 ± 0.1	0.30 ± 0.2
C17:0	0.62 ± 0.0^{a}	0.92 ± 0.1^{b}	0.77 ± 0.0^{c}	0.64 ± 0.0^{a}	0.93 ± 0.0^{ab}	0.75 ± 0.0^{b}	0.90 ± 0.1^{ab}	0.98 ± 0.1^{a}
C17:1 (n-7)	0.17 ± 0.0^{a}	0.32 ± 0.0^{b}	0.15 ± 0.0^{a}	0.13 ± 0.0^{a}	0.01 ± 0.0	ND	0.27 ± 0.2	0.02 ± 0.0
C18:0	38.78 ± 0.8^{a}	41.78 ± 0.2^{b}	41.10 ± 0.2^{ab}	40.05 ± 0.5^{ab}	12.96 ± 0.4	12.27 ± 0.1	15.44 ± 1.5	14.55 ± 1.1
C18:1 (n-7 + n-9)	11.08 ± 0.4^{a}	12.75 ± 0.1^{b}	8.58 ± 0.2^{c}	7.22 ± 0.2^{d}	1.88 ± 0.1	2.02 ± 0.2	2.39 ± 0.2	1.86 ± 0.1
C18:2 (n-6)	$\mathbf{30.68 \pm 1.1^{a}}$	$\mathbf{1.63 \pm 0.1^{b}}$	$\mathbf{29.89 \pm 0.6^{a}}$	$\mathbf{37.08 \pm 0.2^{c}}$	$\mathbf{4.72 \pm 0.4^{a}}$	$\mathbf{1.07 \pm 0.1^{b}}$	$\mathbf{5.92 \pm 1.1^{a}}$	$\mathbf{6.00 \pm 0.1^{a}}$
C18:3 (n-3)	0.02 ± 0.0^{a}	0.13 ± 0.0^{b}	ND	ND	ND	ND	0.02 ± 0.0	0.12 ± 0.1
C20:0	0.86 ± 0.1^{a}	0.55 ± 0.0^{b}	0.60 ± 0.0^{bc}	0.67 ± 0.0^{c}	1.16 ± 0.0	1.40 ± 0.1	1.24 ± 0.1	1.13 ± 0.0
C20:1 (n-7 + n-9)	0.22 ± 0.0^{a}	0.06 ± 0.0^{bc}	0.02 ± 0.0^{c}	0.13 ± 0.0^{b}	ND	ND	ND	ND
C20:2 (n-6)	1.36 ± 0.1^{a}	0.02 ± 0.0^{b}	0.45 ± 0.0^{c}	1.53 ± 0.0^{a}	0.71 ± 0.2^{a}	ND	0.24 ± 0.0^{b}	0.72 ± 0.1^{a}
C20:3 (n-6)	0.30 ± 0.1^{a}	0.81 ± 0.0^{b}	0.27 ± 0.0^{a}	0.18 ± 0.0^{c}	ND	ND	0.03 ± 0.0	ND
C20:4 (n-6)	$\mathbf{0.69 \pm 0.1^{a}}$	$\mathbf{0.65 \pm 0.1^{a}}$	$\mathbf{0.33 \pm 0.0^{b}}$	$\mathbf{0.36 \pm 0.1^{b}}$	$\mathbf{0.35 \pm 0.0^{a}}$	$\mathbf{0.31 \pm 0.0^{ab}}$	$\mathbf{0.25 \pm 0.0^{ab}}$	$\mathbf{0.24 \pm 0.0^{b}}$
C20:5 (n-3)	$\mathbf{0.14 \pm 0.0^{a}}$	$\mathbf{5.55 \pm 0.5^{b}}$	$\mathbf{1.18 \pm 0.1^{c}}$	$\mathbf{0.18 \pm 0.0^{a}}$	$\mathbf{0.34 \pm 0.0^{a}}$	$\mathbf{2.58 \pm 0.3^{b}}$	$\mathbf{1.22 \pm 0.0^{c}}$	$\mathbf{0.48 \pm 0.0^{a}}$
C22:0	0.88 ± 0.1^{a}	0.46 ± 0.0^{b}	0.49 ± 0.0^{bc}	0.59 ± 0.0^{c}	5.83 ± 0.2^{ab}	7.67 ± 0.3^{b}	6.13 ± 0.6^{ab}	5.11 ± 0.4^{a}
C22:2 (n-6)	0.49 ± 0.0^{ab}	0.16 ± 0.2^{b}	0.37 ± 0.0^{ab}	0.66 ± 0.1^{a}	1.59 ± 0.1^{a}	0.47 ± 0.0^{b}	1.13 ± 0.1^{a}	2.21 ± 0.0^{c}
C22:4 (n-6)	0.85 ± 0.1	0.86 ± 0.1	0.56 ± 0.2	0.49 ± 0.1	0.83 ± 0.3	0.44 ± 0.0	0.48 ± 0.0	0.31 ± 0.0
C22:5 (n-3)	$\mathbf{0.95 \pm 0.1^{a}}$	$\mathbf{14.36 \pm 0.6^{b}}$	$\mathbf{3.13 \pm 0.1^{c}}$	$\mathbf{0.64 \pm 0.1^{d}}$	**ND**	$\mathbf{0.94 \pm 0.1^{b}}$	$\mathbf{0.53 \pm 0.1^{c}}$	$\mathbf{0.10 \pm 0.1^{a}}$
C24:0	0.39 ± 0.1	0.27 ± 0.0	0.28 ± 0.1	0.24 ± 0.0	6.18 ± 0.2	7.15 ± 0.2	6.33 ± 0.7	5.45 ± 0.5
C22:6 (n-3)	$\mathbf{0.81 \pm 0.1^{a}}$	$\mathbf{6.27 \pm 0.6^{b}}$	$\mathbf{1.65 \pm 0.1^{c}}$	$\mathbf{0.44 \pm 0.1^{d}}$	**ND**	$\mathbf{0.84 \pm 0.1^{b}}$	$\mathbf{0.26 \pm 0.1^{a}}$	$\mathbf{0.10 \pm 0.1^{a}}$
C24:1 (n-9)	0.13 ± 0.0^{a}	ND	0.02 ± 0.0^{b}	0.07 ± 0.0^{ab}	10.17 ± 0.6	9.65 ± 1.0	9.47 ± 0.5	9.20 ± 0.5
Σ n-3 PUFA	1.92 ± 0.1^{a}	26.34 ± 1.1^{b}	5.96 ± 0.2^{c}	1.26 ± 0.0^{d}	0.34 ± 0.0^{a}	4.36 ± 0.4^{b}	2.05 ± 0.2^{c}	0.85 ± 0.3^{ac}
Σ n-6 PUFA	32.54 ± 1.2^{a}	4.12 ± 0.1^{b}	31.05 ± 0.3^{a}	38.11 ± 0.3^{c}	5.90 ± 0.3^{a}	1.82 ± 0.1^{b}	6.94 ± 0.9^{a}	6.55 ± 0.2^{a}
Σ SFA	51.14 ± 0.9^{a}	55.25 ± 1.0^{b}	52.81 ± 0.3^{ab}	50.32 ± 0.4^{a}	79.10 ± 1.0^{ab}	81.61 ± 1.0^{a}	77.18 ± 1.2^{b}	78.30 ± 0.2^{ab}
Σ MUFA	12.55 ± 0.5^{a}	14.11 ± 0.1^{b}	9.35 ± 0.2^{c}	8.12 ± 0.2^{c}	12.36 ± 0.5	11.73 ± 0.7	12.45 ± 0.3	11.37 ± 0.3
Σ PUFA	36.31 ± 1.3^{a}	30.64 ± 1.0^{b}	37.84 ± 0.2^{a}	41.56 ± 0.3^{c}	8.54 ± 0.5^{ab}	6.65 ± 0.5^{b}	10.37 ± 1.0^{a}	10.33 ± 0.3^{a}

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4. DEVELOPMENT OF A MICROSCALE METHOD FOR THE ISOLATION OF LIPID RAFTS FROM HUMAN BREAST CANCER CELLS

4.1 INTRODUCTION

Recent findings in membrane biology suggest that the plasma membrane is composed of microdomains of saturated lipids that segregate together to form lipid "rafts". These lipid rafts are rich in saturated fatty acids, sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins (Simons and Ikonen, 1997). Lipid rafts are operationally defined as cholesterol-dependent membrane microdomains resistant to solubilization by non-ionic detergents at low temperatures, and can be isolated from most mammalian cell types (Brown and London, 1998). A number of proteins involved in signaling have been found in rafts (Foster *et al.*, 2003), thus these specialized regions within the plasma membrane are thought to play a role in cell signal transduction, perhaps by serving as platforms to facilitate the association of signaling molecules (Simons and Toomre, 2000).

Different methods have been used for the study of membrane lipid rafts, the most popular being raft isolation by density gradient centrifugation of cell lysates prepared with non-ionic detergents (eg. Triton X-100, Brij, CHAPS, NP-40). Lipid rafts are insoluble in non-ionic detergents at 4 °C, and because of their high lipid content, they will float to a low density during gradient centrifugation (Simons and Ikonen, 1997). Current density centrifugation protocols typically involve large ultracentrifuge tubes and volumes of reagents, necessitating the use of larger numbers of cells (eg. 10^8 cells) and longer ultracentrifugation times (eg. 16 to 18 hours) in order to visualize and acquire sufficient raft material for subsequent assays. This chapter describes an adaptation of the raft isolation methods by Brown and Rose (1992) and Li *et al.* (2003) for a tabletop ultracentrifuge. This microscale method uses a significantly smaller volume of reagents, and enables us to isolate lipid rafts from a smaller number of cells, and in about half the time as currently required by large-scale ultracentrifugation protocols.

4.2 MATERIALS AND METHODS

Cell culture

The MDA-MB-231 and MCF-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD). All media and media components were obtained from Gibco Invitrogen Corporation (Burlington, Ontario, Canada) unless otherwise stated. MDA-MB-231 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 1 % v/v antibiotic/antimycotic solution (1 x 10^5 U/L penicillin, 100 mg/L streptomycin, and 25 mg/L amphotericin B) and 5 % v/v fetal calf serum (FCS). MCF-7 cells were grown in minimum essential media (MEM) supplemented with 1 % v/v antibiotic/antimycotic solution and 5 % v/v FCS, 1 mM sodium pyruvate, and 10 µg/mL bovine insulin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Cells were grown at 37 °C in 5 % CO_2 at 98 % relative humidity. Cells were incubated with fatty acids as described in chapter 3.

Lipid raft isolation

This lipid raft isolation protocol was adapted from the method of Brown and Rose (Brown and Rose, 1992), with slight modification. Cells (approximately 2-4 x 10^7) were lysed on ice in 500 µL TNE (25 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with 1 % v/v Triton X-100 (VWR, Edmonton, Alberta, Canada) and freshly added protease and phosphatase inhibitor cocktails added at 1:100 dilution (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Cell lysates were passed through a 26 ½ G needle ten times, and incubated on ice for 30 minutes. Lysates were centrifuged at 1000 rpm (200 x g) for 2 minutes at 4 °C, and the supernatants (500 µL) were transferred to cooled, 5 mL ultracentrifuge tubes (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada). Lysates were mixed with 500 µL of 80 % w/v sucrose (in TNE), and overlaid with 2 mL of 30 % w/v sucrose (in TNE) and then 2 mL of 5 % w/v sucrose (in TNE). Tubes were centrifuged at 50,000 rpm for 8 hours at 4 °C in an Optima™ Max Ultracentrifuge, MLS-50 rotor (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada). This centrifugation time was adapted from the method of Li *et al.* (2003) whose protocol involved a centrifugation speed of 37,000 rpm for 17 h in a Beckman XL-70 ultracentrifuge with an SW41 Ti rotor (see calculation, Figure 4-1). Following centrifugation, six fractions (800 μL each) were collected sequentially from the top of the gradient to the bottom, and stored at –80 °C until further assay.

Characterization of sucrose gradient fractions

To confirm the location of lipid rafts on the gradients, lipid raft fractions were dot-blotted onto nitrocellulose membranes and probed for the presence of the raft markers GM1 and $G_{\alpha i}$. $G_{\alpha i}$ is a heterotrimeric G protein subunit known to reside in rafts (Moffett *et al.*, 2000), whereas GM1 is a ganglioside also enriched in rafts and commonly used as a raft marker (Stulnig *et al.*, 1998; Kalka *et al.*, 2001). Aliquots (10 μL) of each fraction were spotted in duplicate directly onto nitrocellulose membranes. Membranes were blocked for one hour in TBST (10 mM TrisHCl pH 7.4, 150 mM NaCl, 0.1 % v/v Tween-20) containing 5 % w/v powdered milk, and then probed for $G_{\alpha i}$ (anti-$G_{\alpha i}$; Oncogene Research Products; CedarLane Laboratories Ltd., Hornby, Ontario, Canada, diluted 1:500), and GM1 (using HRP-conjugated cholera toxin B subunit, Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada, diluted 1:12,000) for one hour at room temperature in TBST containing 1 % w/v powdered milk. Membranes stained for $G_{\alpha i}$ were washed three times with TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody (BD Transduction Laboratories™, Mississauga, Ontario, Canada) diluted 1:1000 in TBST containing 1 % w/v powdered milk for one hour at room temperature. Membranes were washed five times and then developed using an enhanced chemiluminescence (ECL™) detection kit (Amersham Biosciences, Baie D'Urfe, Quebec, Canada) and Hyperfilm™ ECL™ film (Amersham Biosciences, Baie D'Urfe, Quebec, Canada).

4.3 RESULTS

Both $G_{\alpha i}$ and GM1 stained intensely in fraction 3 which included the 5 %/30 % interface where flocculent material could be seen. There was some solubilization of $G_{\alpha i}$, which was found in the sixth fraction, but this was not unexpected, as Triton X-100 is known to solubilize some raft protein (Dr. Julie Deans, University of Calgary, personal communication). The same pattern of $G_{\alpha i}$ and GM1 staining was observed in all fatty acid treatments (Figure 4-2). Based on these data, for subsequent experiments, the visible, flocculent material at the 5 %/30 % interface encompassing fraction 3 (approx. 1 mL) was collected and referred to as the lipid raft-enriched fraction.

4.4 SUMMARY

This chapter describes the development of a microscale method for the isolation of lipid rafts from human mammary tumor cells by sucrose gradient density centrifugation that was successfully adapted from published raft isolation methods. This microscale method saves time (due to the significantly shorter centrifugation period as compared to current protocols), and utilizes fewer cells. The isolation of lipid rafts by this method was not affected by polyunsaturated fatty acid treatment, and this method will be used in subsequent experiments for the study of lipid rafts.

Formula:

$$\frac{(rpm_1)^2 \text{ x } t_1 = \ln(r_{max}/r_{min})_1}{(rpm_2)^2 \text{ x } t_2 = \ln(r_{max}/r_{min})_2}$$

Variables[1]*:*

$rpm_1 = 37{,}000$ $rpm_2 = 50{,}000$
$r_{max1} = 153.1$ mm $r_{max2} = 95.8$ mm
$r_{min1} = 67.4$ mm $r_{min2} = 47.5$ mm
$t_1 = 17$ hours

Calculation for t_2:

$$\frac{(37{,}000)^2 \text{ x } 17 = \ln(153.1/67.4)}{(50{,}000)^2 \text{ x } t_2 = \ln(95.8/47.5)} \qquad \underline{\underline{t_2 = 7.96 \text{ hours}}}$$

Figure 4-1 Calculation of ultracentrifugation run time. The ultracentrifugation time for the current protocol (t_2) was adapted from the centrifugation specifications of Li *et al.* (2003), using the above formula. [1]The rpm_1 (37,000) and t_1 (17 hours) are from Li *et al.*; rpm_2 (50,000) is the maximum rpm of the Optima™ Max Ultracentrifuge, MLS-50 rotor used in the current study. The r_{max1} and r_{min1} are specific to the SW41 Ti rotor used by Li *et al.*, whereas the r_{max2} and r_{min2} are specific to the MLS-50 rotor used in the current study.

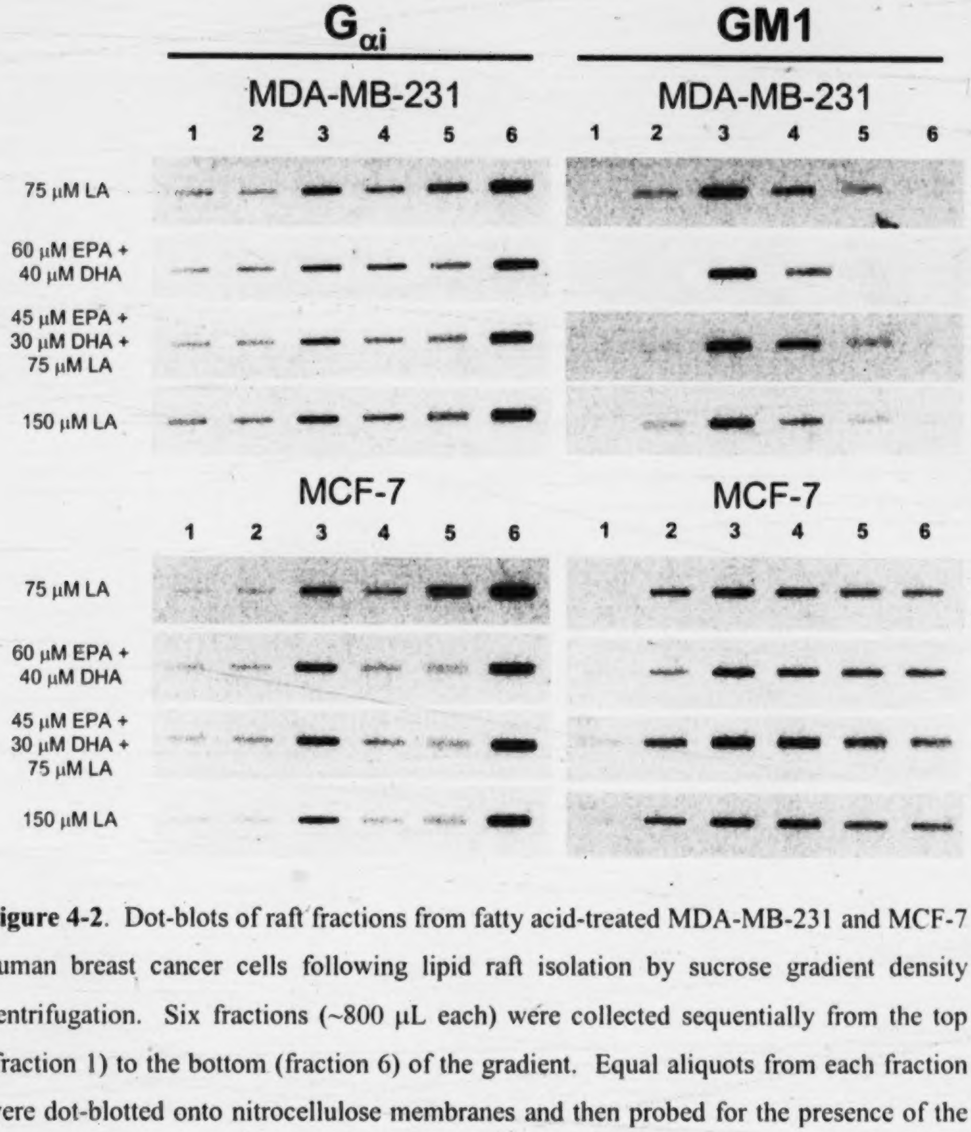

Figure 4-2. Dot-blots of raft fractions from fatty acid-treated MDA-MB-231 and MCF-7 human breast cancer cells following lipid raft isolation by sucrose gradient density centrifugation. Six fractions (~800 μL each) were collected sequentially from the top (fraction 1) to the bottom (fraction 6) of the gradient. Equal aliquots from each fraction were dot-blotted onto nitrocellulose membranes and then probed for the presence of the raft markers $G_{\alpha i}$ (left column) and GM1 (right column).

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5. OMEGA-3 POLYUNSATURATED FATTY ACIDS ALTER MEMBRANE RAFT LIPID AND PROTEIN COMPOSITION IN BREAST CANCER CELLS[1,2]

5.1 INTRODUCTION

The inhibitory effects of the long-chain polyunsaturated fatty acids, EPA and DHA, on mammary tumor development have been well documented in rodent models (see Rose and Connolly, 1999b for review) and in human breast cancer cell lines (Chapter 3 and Begin *et al.*, 1986; Begin *et al.*, 1988; Rose and Connolly, 1990; Grammatikos *et al.*, 1994; Chajes *et al.*, 1995; Noguchi *et al.*, 1995; Abdi-Dezfuli *et al.*, 1997; Senzaki *et al.*, 1998; Yamamoto *et al.*, 1999; Chamras *et al.*, 2002). Despite these observations, the mechanism(s) for how n-3 PUFA inhibit breast cancer cell growth are not well understood. N-3 PUFA are significantly incorporated into tumor cell membrane phospholipids when provided in the diet (see Jurkowski and Cave, 1985; Karmali *et al.*, 1989; Rose *et al.*, 1995; Robinson *et al.*, 2002) or in the culture media (Chapter 3), but the biological impact of this has not been established. Based on work in other cells and cell lines, these fatty acids may exert their growth-inhibitory effects on tumor cells by altering plasma membrane conditions or associated signaling events (Field and Schley, 2004).

Recent findings in membrane biology suggest that the plasma membrane is composed of microdomains of saturated lipids that segregate together to form lipid "rafts". These lipid rafts are rich in saturated fatty acids, sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins (Simons and Ikonen, 1997). Lipid rafts are operationally defined as cholesterol-dependent membrane microdomains resistant to solubilization by non-ionic detergents at low temperatures, and can be isolated from nearly all mammalian cell types (Brown and London, 1998). A subset of specialized rafts termed caveolae also have been described, which are flask-shaped invaginations in the membrane that are enriched in the integral membrane protein caveolin, and are believed

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[1] A version of this chapter has been submitted for publication to the *Journal of Biological Chemistry*.

[2] This work was presented in part at Canadian Federation of Biological Societies, June 2004, Vancouver, BC and published in part in abstract form. (Schley, P.D. and Field, C.J. N-3 fatty acids are incorporated into and displace signaling proteins from lipid rafts in human breast cancer cells. CFBS 47: F25).

to have several functions including endocytosis and cholesterol transport (Ma *et al.*, 2004b). A number of proteins involved in signaling have been found in rafts (Foster *et al.*, 2003), thus these specialized regions within the plasma membrane are thought to play a role in cell signal transduction, perhaps by serving as platforms to facilitate the association of signaling molecules (Simons and Toomre, 2000).

The epidermal growth factor receptor (EGFR) is one of many signaling molecules believed to associate with rafts. While early reports suggested that both inactive and phosphorylated forms of the EGFR were located within caveolae/lipid rafts (Mineo *et al.*, 1996; Waugh *et al.*, 1999), accumulating evidence suggests that caveolae/rafts function as negative regulators of EGFR phosphorylation (Pike and Casey, 2002; Roepstorff *et al.*, 2002; Takebayashi *et al.*, 2004), with ligand binding and receptor activation leading to a migration of the EGFR out of caveolae/rafts (Mineo *et al.*, 1999; Abulrob *et al.*, 2004).

Signaling through the EGFR as well as a number of other growth factor receptors leads to the activation of a number of other key signaling molecules, including phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), and Akt. The activation of phospholipase C enzymes is a ubiquitous signaling event triggered by hormone and growth factor receptors (Carpenter and Ji, 1999). PLCγ isoforms (PLCγ1 and PLCγ2) in particular are mainly regulated by receptors with intrinsic tyrosine kinase activity or linked to non-receptor tyrosine kinases (Katan *et al.*, 2003). Most polypeptide growth factor receptors with intrinsic tyrosine kinase activity have been linked to the activation of PLCγ (Katan, 1998). The mechanism of PLCγ activation involves its association with and phosphorylation by receptor and non-receptor tyrosine kinases. Activated PLCγ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), eventually leading to an increase in intracellular Ca^{2+} levels and protein kinase C (PKC) activation. PLCγ-mediated signaling is believed to play a role in cell motility and proliferation (Wells and Grandis, 2003). Targeting of PLCγ to the plasma membrane is critical for its activation, and there is some evidence that PLCγ activation may occur via recruitment to lipid rafts (Veri *et al.*, 2001; Wang *et al.*, 2001; Hur *et al.*, 2004).

PI3K plays a central role in cellular signaling, regulating a number of biological responses including proliferation, apoptosis, differentiation, and chemotaxis. PI3K consists of a 110-kDa catalytic subunit (of which there are α, β, and δ isoforms) associated with an 85-kDa regulatory subunit (with α and β isoforms, as well as a 55-kDa splice variant) (Vivanco and Sawyers, 2002). The regulatory subunits have no enzymatic activity, but function to recruit the catalytic subunit to specific cellular locations such as the plasma membrane (Xia *et al.*, 2003). Like PLCγ, the regulatory subunit of PI3K is activated through its association with cytoplasmic and receptor tyrosine kinases, either directly or through intermediate adaptor proteins (Vivanco and Sawyers, 2002). PI3K then catalyses the phosphorylation of phosphatidylinositol lipids, mainly PIP_2, to synthesize PIP_3. Akt (also known as protein kinase B) is then recruited to the plasma membrane through direct contact with PIP_3, where it is activated, and mediates cellular survival (Vivanco and Sawyers, 2002). Recent studies suggest that Akt activation is dependent upon the integrity of lipid rafts (Zhuang *et al.*, 2002; Bauer *et al.*, 2003; Ha *et al.*, 2003; Elhyany *et al.*, 2004). While PI3K has been detected in rafts (Lang *et al.*, 2002; Peres *et al.*, 2003), less is known about the role of rafts in PI3K signaling.

Relatively little is known at this point about the effects of n-3 PUFA on the lipid and protein composition of membrane rafts. To our knowledge, the impact of n-3 PUFA on lipid rafts in tumor cells has not been examined. In this chapter, it is shown that n-3 PUFA are significantly incorporated into, and alter the phospholipid content, of membrane rafts in two human breast cancer cell lines, MDA-MB-231 and MCF-7. Furthermore, n-3 PUFA treatment alters the protein composition of lipid rafts, decreasing EGFR protein levels in lipid rafts of MDA-MB-231 cells. There was also a trend toward decreased phospholipase Cγ and increased PI3K protein levels in lipid rafts of n-3 PUFA-treated MDA-MB-231 cells. Displacement of the EGFR from rafts by both n-3 PUFA treatments is associated with an increase in EGFR tyrosine phosphorylation. These results suggest that incorporation of n-3 PUFA into membrane raft lipids has significant effects on the lipid composition of rafts in human breast cancer cells, and this is associated with changes in the distribution and function of important growth signaling molecules within lipid rafts.

5.2 MATERIALS AND METHODS

Cell culture

The MDA-MB-231 and MCF-7 cell lines were obtained and cultured as described in chapter 3.

Fatty acids

EPA, DHA and LA were obtained and stored as described in chapter 3.

Preparation of fatty acid-supplemented media

Fatty acid-supplemented media was prepared as described in chapter 3, with the following modification: in the growth experiments conducted to obtain lipid rafts, the media was formulated to contain either 4 % v/v FCS (MDA-MB-231) or 4.5 % v/v FCS (MCF-7).

Growth experiments

For lipid raft isolation, cells were seeded at 3 x 10^6 (MDA-MB-231) or 4 x 10^6 (MCF-7) cells per flask (150 cm^2) in media containing 4 % (MDA-MB-231) or 4.5 % (MCF-7) v/v FCS and allowed 48 h to adhere. After 48 h, the culture media was replaced with fresh media containing the experimental fatty acids. The cells were incubated for 72 h in the presence of fatty acids, during which time the media was not changed. After 72 h, the cells were harvested using trypsin-EDTA (Gibco Invitrogen Corporation, Burlington, Ontario, Canada), and viable cells were counted under a microscope by trypan blue (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) exclusion. Cell counts for each fatty acid treatment were converted to a percentage of the control treatment, 75 μM LA.

Separate growth experiments were conducted to obtain cells for whole cell fatty acid composition, ceramide quantification, western blotting and phosphorylated EGFR determination. For both cell lines, cells were seeded at 1 x 10^6 cells per flask (75 cm^2) in media containing 5 % v/v FCS, and growth experiments were conducted as described above.

Lipid raft isolation

Lipid rafts were isolated from tumor cell membranes as described in chapter 4. To confirm the location of lipid rafts on the gradients, six fractions (800 μL each) were collected sequentially from the top of the gradient to the bottom, and 10 μL aliquots of each fraction were dot-blotted directly onto nitrocellulose membranes. Membranes were probed for the presence of the raft markers $G_{\alpha i}$ (using an anti-$G_{\alpha i}$ antibody, Oncogene Research Products; CedarLane Laboratories Ltd., Hornby, Ontario, Canada) and GM1 (using HRP-conjugated cholera toxin B subunit, Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). As described in chapter 4, both $G_{\alpha i}$ and GM1 stained most intensely in fraction 3, which included the 5 %/30 % interface where flocculent material could be seen. This same pattern was observed in all fatty acid treatments. Based on these data, for subsequent experiments, the visible, flocculent material at the 5 %/30 % interface encompassing fraction 3 (approx. 1 mL) was collected and referred to as the lipid raft-enriched fraction.

Fatty acid composition analysis

Lipids from whole cells or pelleted rafts were extracted using a modified Folch procedure (Folch *et al.*, 1957) as previously described (Field *et al.*, 1988). For fatty acid composition analysis of lipid rafts, fatty acid methyl esters of the extracted raft lipids were prepared using 14 % w/v BF_3/methanol reagent (Morrison and Smith, 1964), and separated by automated gas liquid chromatography (Varian CP-3800, Varian Inc., Mississauga, Ontario, Canada) on an SGE BP20 column (60 m x 0.25 mm internal diameter; Varian Inc., Mississauga, Ontario, Canada). For whole cell fatty acid composition, total phospholipid was isolated from samples spotted on silica gel 'G' thin layer chromatography plates (20 x 20 cm, Analtek, Newark, DE, USA) and developed in petroleum ether : diethyl ether : acetic acid (80 : 20 : 1, by vol.) for approximately 30 minutes. The total phospholipid band (at the origin) was visualized with 8-anilino-1-naphthalene-sulfonic acid under ultraviolet light. Fatty acid methyl esters from the total phospholipid band were prepared using 14 % w/v BF_3/methanol reagent (Morrison and Smith, 1964), and separated by automated gas liquid chromatography as described above.

Phosphorus assay

Lipids from pelleted rafts were extracted as above, and individual phospholipid classes were separated on thin layer chromatography plates (HPK silica gel 60 nm 10 x 10 cm; Whatman, Clifton, NJ) as previously described (Touchstone *et al.*, 1980). Separated phospholipids were visualized with 8-anilino-1-naphthalene-sulfonic acid and identified under ultraviolet light with appropriate standards (Supelco Canada Ltd., Oakville, Ontario, Canada). Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin bands were scraped into phosphate-free test tubes. Phosphorus was determined by a modified Bartlett assay for microscale lipid phosphorus analysis (Itoh *et al.*, 1986). Absorbance was measured at 790 nm with a Molecular Devices SpectraMax PLUS 384 spectrophotometer (Sunnyvale, CA). Phospholipid phosphorus content was determined by reference to phosphate standards (1.5 mM KH_2PO_4), and expressed as a percentage of the total phospholipid phosphorus.

Ceramide quantitation

Lipids were extracted from fatty acid-treated MDA-MB-231 cells and ceramide levels were quantified by a DAG kinase assay as described by Payne *et al.* (1999). Briefly, ceramide was enzymatically converted to ceramide-1-phosphate with [γ^{32}P]ATP by DAG kinase. Ceramide-1-phosphate was separated by thin-layer chromatography, and the mass of ceramide was calculated after scintillation counting and reference to appropriate standard curves.

Western blotting

Whole cells or pelleted rafts were resuspended in lysis buffer (20 mM TrisHCl pH 7.4, 137 mM NaCl, 10 % v/v glycerol, 1 % v/v Nonidet P-40, 2 mM EDTA) with freshly added protease and phosphatase inhibitor cocktails (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), and the protein concentration of the lysates was determined using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Equal amounts of protein from each treatment were separated by SDS-PAGE on 10 % polyacrylamide gels. Rainbow^{TM} (Amersham Biosciences, Baie D'Urfe, Quebec, Canada) colored molecular weight standards were used to monitor protein separation. Proteins were electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Baie D'Urfe, Quebec, Canada). Even protein loading and transfer was confirmed by staining with Ponceau S (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Membranes were blocked for one hour at room temperature with TBST (10 mM TrisHCl pH 7.4, 150 mM NaCl, 0.1 % v/v Tween-20) and 5 % w/v powdered milk. Primary antibodies to EGFR, Akt, PLCγ1 and PI3K (p85 subunit) (Cell Signaling Technology, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) were diluted 1:500 in TBST containing 5 % w/v bovine serum albumin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and incubated with membranes overnight at 4 °C. Membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (BD Transduction Laboratories™, Mississauga, Ontario, Canada) diluted 1:500 in TBST containing 1 % w/v powdered milk for one hour at room temperature. Membranes were developed using an enhanced chemiluminescence (ECL™) detection kit (Amersham Biosciences, Baie D'Urfe, Quebec, Canada) and Hyperfilm™ ECL™ film (Amersham Biosciences, Baie D'Urfe, Quebec, Canada). The relative intensities of band signals were determined using a Bio-Rad GS-670 imaging densitometer (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada).

EGFR phosphorylation

For analysis of EGFR phosphorylation, fatty acid-treated cells were stimulated with 50 ng/mL epidermal growth factor (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) for 30 minutes at 37 °C prior to harvesting with trypsin-EDTA. EGFR phosphorylation was quantified using an Active EGF Receptor ELISA kit (Calbiochem, CedarLane Laboratories Ltd., Hornby, Ontario, Canada) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were conducted using the SAS statistical package, version 8 (SAS Institute, Cary, NC). Data sets that were not normally distributed were log-transformed prior to statistical analysis. Data were analyzed by one-way ANOVA, blocked by experiment/passage number, followed by a Tukey test to identify significant ($p<0.05$) differences between fatty acid treatments.

5.3 RESULTS

EPA and DHA decrease the growth of human breast cancer cells

Treatment of MDA-MB-231 and MCF-7 cells with n-3 PUFA resulted in a significantly lower number of viable cells after 72 hours compared to control-treated cells (Figure 5-1). A mixture of EPA and DHA (60 μM EPA + 40 μM DHA) significantly ($p<0.05$) decreased cell population growth by 50 and 64 % in MCF-7 and MDA-MB-231 cells, respectively, compared to the control treatment, 75 μM LA. Incubation of cells with n-3 PUFA in the presence of LA (i.e. 45 μM EPA + 30 μM DHA + 75 μM LA) significantly ($p<0.05$) decreased the number of cells per flask by 44 and 54 % in MCF-7 and MDA-MB-231 cells, respectively, compared to incubation with 75 μM LA alone, confirming that growth inhibition by EPA and DHA could still be achieved in the presence of abundant LA and was not due to LA deprivation. This decrease in cell population growth also was not due to a cytotoxic effect of increased fatty acid concentration in the media (150 μM) as incubation with 150 μM LA did not significantly affect growth in either cell line.

EPA and DHA alter lipid raft fatty acid composition in human breast cancer cells

MDA-MB-231. Concentrations of EPA (C20:5n-3), DPA (C22:5n-3), DHA (C22:6n-3), and total n-3 PUFA were significantly ($p<0.05$) increased in lipid rafts as well as whole cell phospholipids following treatment with either 60 μM EPA + 40 μM DHA or 45 μM EPA + 30 μM DHA + 75 μM LA, as compared to treatment with 75 or 150 μM LA (Table 5-1). Enrichment of EPA, DPA, DHA and total n-3 PUFA was significantly lower with 45 μM EPA + 30 μM DHA + 75 μM LA versus 60 μM EPA + 40 μM DHA treatment in both rafts and whole cell phospholipids. Cells treated with 60 μM EPA + 40 μM DHA, but not 45 μM EPA + 30 μM DHA + 75 μM LA, had a significantly lower concentration of LA (C18:2n-6) compared to treatment with 75 μM LA in both rafts and whole cell phospholipids. Both n-3 PUFA treatments significantly decreased the concentration of AA (C20:4n-6) in rafts and whole cell phospholipids relative to treatment with 75 µM LA. The total n-6 PUFA concentration was significantly decreased with 60 µM EPA + 40 µM DHA treatment, but not 45 µM EPA + 30 µM DHA + 75 µM LA treatment, in rafts relative to 75 µM LA treatment. Treatment with 60 µM EPA + 40 µM DHA significantly increased the concentration of palmitic acid (C16:0) in both rafts and whole cell phospholipids relative to treatment with 75 µM LA. The unsaturation index was significantly increased with 60 µM EPA + 40 µM DHA treatment, but not 45 µM EPA + 30 µM DHA + 75 µM LA treatment, in rafts relative to 75 µM LA treatment. In general, lipid rafts had a lower concentration of both n-3 and n-6 PUFA and a lower unsaturation index, as well as a higher concentration of SFA, relative to whole cell phospholipids.

MCF-7. Concentrations of EPA, DPA, DHA, and total n-3 PUFA were significantly ($p<0.05$) increased in lipid rafts as well as whole cell phospholipids following treatment with either 60 µM EPA + 40 µM DHA or 45 µM EPA + 30 µM DHA + 75 µM LA, as compared to treatment with 75 or 150 µM LA (Table 5-2). Enrichment of EPA, DPA, DHA and total n-3 PUFA was significantly lower with 45 µM EPA + 30 µM DHA + 75 µM LA versus 60 µM EPA + 40 µM DHA treatment in both rafts and whole cell phospholipids. Cells treated with 60 µM EPA + 40 µM DHA, but not 45 µM EPA + 30 µM DHA + 75 µM LA, had a significantly lower concentration of LA compared to treatment with 75 µM LA in both rafts and whole cell phospholipids. Treatment with 45 µM EPA + 30 µM DHA + 75 µM LA or 150 µM LA significantly decreased the concentration of AA as compared to treatment with 75 µM LA in both rafts and whole cell phospholipids. The total n-6 PUFA concentration was significantly decreased with 60 µM EPA + 40 µM DHA treatment, but not 45 µM EPA + 30 µM DHA + 75 µM LA treatment, in rafts relative to 75 µM LA treatment. Treatment with 60 µM EPA + 40 µM DHA significantly increased the concentration of palmitic acid (C16:0) in both rafts and whole cell phospholipids relative to treatment with 75 µM LA. The total SFA concentration was significantly increased, whereas the total PUFA concentration was significantly decreased, with 60 µM EPA + 40 µM DHA treatment relative to 75 µM LA treatment in rafts as well as whole cell phospholipids. In general, lipid rafts had a lower concentration of both n-3 and n-6 PUFA and a lower unsaturation index, as well as a higher concentration of SFA, relative to whole cell phospholipids.

EPA and DHA alter the phospholipid composition of lipid rafts in human breast cancer cells

A mixture of EPA and DHA (60 μM EPA + 40 μM DHA) significantly ($p<0.05$) reduced the amount (% of the total phospholipids) of sphingomyelin (SM) and increased the amount of phosphatidylcholine (PC) in rafts from both breast cancer cell lines (Table 5-3). There was a trend toward reduced SM with 45 μM EPA + 30 μM DHA + 75 μM LA treatment, but the difference did not reach statistical significance.

EPA and DHA do not alter ceramide levels in MDA-MB-231 cells

There was no effect of EPA and DHA treatment on whole cell levels of ceramide in MDA-MB-231 cells (Table 5-4).

EPA and DHA decrease EGFR protein levels in lipid rafts and increase whole cell EGFR phosphorylation in MDA-MB-231 cells

Treatment with n-3 PUFA resulted in a marked decrease in EGFR protein levels in lipid rafts of MDA-MB-231 cells (Figure 5-2). The lower concentration of the EGFR in lipid rafts was not the result of a lower cellular expression of the EGFR, as total cellular EGFR protein levels remained unchanged by n-3 PUFA treatment (Figure 5-2). The amount of phosphorylated EGFR (in whole cells) was significantly ($p<0.05$) increased by both 60 μM EPA + 40 μM DHA and 45 μM EPA + 30 μM DHA + 75 μM LA treatments as compared to LA-treated cells (Figure 5-3).

EPA and DHA may affect the raft distribution of PLCγ and PI3K in MDA-MB-231 cells

Treatment with n-3 PUFA appeared to reduce the amount of phospholipase Cγ in lipid rafts in MDA-MB-231 cells relative to control-treated cells (Figure 5-4). Our antibody to the p85 regulatory subunit of PI3K recognized a band of ~ 55 kDa (in raft samples as well as whole cell lysates), suggesting that the p55 regulatory subunit of PI3K predominates in this cell line. Levels of this p55 PI3K appeared higher in lipid rafts of n-3 PUFA-treated cells versus that of control-treated cells (Figure 5-4). Akt levels within lipid rafts did not appear to be altered with EPA and DHA treatment (Figure 5-4).

5.4 DISCUSSION

We have demonstrated that incubating both MDA-MB-231 and MCF-7 human breast cancer cells with EPA and DHA significantly inhibits tumor cell population growth (or expansion), as estimated by a decrease in viable cell numbers. This finding is in agreement with the existing literature demonstrating that n-3 PUFA inhibit breast cancer cell growth *in vitro* (Begin *et al.*, 1986; Begin *et al.*, 1988; Rose and Connolly, 1990; Grammatikos *et al.*, 1994; Chajes *et al.*, 1995; Noguchi *et al.*, 1995; Abdi-Dezfuli *et al.*, 1997; Senzaki *et al.*, 1998; Yamamoto *et al.*, 1999; Chamras *et al.*, 2002) and *in vivo* (Karmali *et al.*, 1984; Braden and Carroll, 1986; Gonzalez *et al.*, 1993; Connolly *et al.*, 1997; Rose and Connolly, 1999a). In addition, our inclusion of a fatty acid treatment consisting of a mixture of EPA and DHA along with LA demonstrates that the growth-inhibitory effects of n-3 PUFA on breast cancer cells are still achieved in the presence of abundant LA, a limitation of many of the previous studies.

Although it is well documented that n-3 PUFA are readily incorporated into tumor cell membranes *in vitro* and *in vivo* (Chapter 3, and Jurkowski and Cave, 1985; Karmali *et al.*, 1989; Grammatikos *et al.*, 1994; Hatala *et al.*, 1994; Rose *et al.*, 1995; Bardon *et al.*, 1996; Robinson *et al.*, 2002), the biological impact of this has not been established. We hypothesized that n-3 PUFA alter the properties of membrane lipid rafts, which have been implicated in signal transduction, as a mechanism to explain their growth-inhibitory effects on tumor cells.

The effects of culturing breast tumor cells with n-3 PUFA on lipid rafts have not been established. Our data demonstrates a significant enrichment of both EPA and DHA into rafts of both breast cancer cell lines treated with 60 μM EPA + 40 μM DHA, and a significant enrichment of both EPA and DHA into rafts of MDA-MB-231 cells treated with 45 μM EPA + 30 μM DHA + 75 μM LA. This is consistent with other recent reports demonstrating a significant incorporation of n-3 PUFA into lipid rafts of different cell types (Jurkat T cells, splenic T cells, colonocytes) when provided *in vitro* and *in vivo* (Stulnig *et al.*, 2001; Fan *et al.*, 2003; Ma *et al.*, 2004a). Similar to the studies by Stulnig *et al.* (2001) and Fan *et al.* (2003), we found that the concentration of EPA and DHA in rafts was less than in whole membrane phospholipids. This was not unexpected considering the high degree of saturation of membrane rafts (as evident in the lower unsaturation index of rafts versus whole membranes); nevertheless, the enrichment of n-3 PUFA in membrane rafts in the current study was quite high (total n-3 PUFA were enriched greater than 12-fold in lipid rafts of MDA-MB-231 cells treated with 60 μM EPA + 40 μM DHA compared to cells treated with 75 μM LA) given the highly saturated environment of rafts. Enrichment of n-3 PUFA was lower in cells treated with 45 μM EPA + 30 μM DHA + 75 μM LA than in cells treated with 60 μM EPA + 40 μM DHA. This was not unexpected due to the lower concentration of n-3 PUFA in the 45 μM EPA + 30 μM DHA + 75 μM LA treatment as well as the inclusion of LA, which would compete with EPA and DHA for membrane phospholipid incorporation (Hatala *et al.*, 1994). Enrichment of n-3 PUFA in membrane phospholipids did not occur at the expense of LA, or total n-6 PUFA, in lipid rafts in either cell line, as treatment with 45 μM EPA + 30 μM DHA + 75 μM LA did not decrease the concentration of LA or total n-6 PUFA relative to that observed with 75 μM LA treatment alone. In the MDA-MB-231 cell line, both n-3 PUFA treatments significantly decreased the AA concentration in rafts and whole cell phospholipids relative to 75 μM LA-treated cells. We did not examine the positional distribution of EPA and DHA within phospholipids, but it is possible that EPA or DHA replaced AA in the sn-2 position. This may have important effects on the activity of certain phospholipases (i.e. PLA_2) which specifically prefer AA in the sn-2 position (Steiner, 1991; Tsegaye *et al.*, 2002; Leslie, 2004).

In the MCF-7 cell line, significant decreases in AA concentrations were only observed with the 45 μM EPA + 30 μM DHA + 75 μM LA and 150 μM LA treatments. This finding is consistent with the effect of PUFA on AA concentrations in individual phospholipids in this cell line as reported in chapter 3. The unsaturation index was significantly increased in lipid rafts of MDA-MB-231 cells treated with 60 μM EPA + 40 μM DHA, which likely reflects the high concentration of n-3 PUFA in these rafts.

Interestingly, in accordance with the findings by Stulnig *et al.* (2001), we also observed an enrichment of saturated fatty acids (primarily palmitic acid, C16:0) in lipid rafts from cells treated with 60 μM EPA + 40 μM DHA. This observation supports the suggestion (Stulnig *et al.*, 2001) that there is a "compensatory mechanism" by which the cells try to maintain raft structures by providing a saturated fatty acyl environment, perhaps through increased fatty acid (i.e. C16:0) synthesis.

Few studies have reported on the phospholipid content of lipid rafts. However, in general, the phospholipid content of mammary tumor cell rafts described in this chapter is similar to that of mouse T cell lipid rafts as described by Fan *et al.* (2003), with PC, followed by SM, being the most abundant phospholipids in rafts, followed by PE, and relatively little PS and PI. Treatment with 60 μM EPA + 40 μM DHA led to a significant decrease in the proportion of SM and a significant increase in the proportion of PC in lipid rafts isolated from both cell lines. Treatment with 45 μM EPA + 30 μM DHA + 75 μM LA reduced the level of raft SM, but the difference did not reach statistical significance. Further research is needed to determine if EPA and DHA in the presence of LA impact on raft phospholipid composition.

Only one other study to our knowledge has examined the effects of n-3 PUFA on the phospholipid content of rafts, and also found a significant decrease in the proportion of SM in rafts isolated from T cells of n-3 PUFA-fed mice (Fan *et al.*, 2003). We hypothesized that the decrease in SM and the increase in PC induced by n-3 PUFA might result from cleavage of SM to generate PC and the lipid second messenger ceramide, which is believed to mediate anti-proliferative responses such as cell cycle arrest, apoptosis and differentiation (Ogretmen and Hannun, 2004). We found no differences in whole cell ceramide levels in MDA-MB-231 cells after fatty acid treatment. However, it is possible that raft ceramide levels may have been altered by n-3 PUFA treatment, which may have local effects, but this effect is not detectable in whole cells. Nevertheless, a decrease in raft SM and an increase in raft PC may have other significance, perhaps affecting the function or structure of lipid rafts, as sphingolipids are required to maintain detergent-insoluble domains in membranes (Schroeder *et al.*, 1998).

The results of this study show a significant effect of n-3 PUFA on lipid raft fatty acid and phospholipid composition. Alterations in the lipid composition of rafts by polyunsaturated fatty acids have been shown to displace signaling proteins from rafts in immune cells (Stulnig *et al.*, 2001; Diaz *et al.*, 2002; Zeyda *et al.*, 2002), indicating that these fatty acids significantly alter raft environment and the function of signaling proteins within rafts. We examined the effects of n-3 PUFA on raft localization of the EGFR, a growth factor receptor that is often overexpressed in breast carcinomas and may be involved in tumor cell progression (Biscardi *et al.*, 1998). MDA-MB-231 cells express high levels of the EGFR compared to MCF-7 cells (which express minimal levels of the receptor) (Biscardi *et al.*, 1998; Alokail, 2004), and thus this cell line was used to study EGFR raft modulation by n-3 PUFA. Our results show that protein levels of the EGFR are significantly reduced in lipid rafts of n-3 PUFA-treated MDA-MB-231 breast cancer cells. In addition, we saw an increase in total cellular EGFR phosphorylation in n-3 PUFA-treated cells (which was significant with 60 μM EPA + 40 μM DHA treatment). This finding is consistent with recent reports in the literature indicating that caveolae/rafts appear to function as negative regulators of EGFR phosphorylation (Pike and Casey, 2002; Roepstorff *et al.*, 2002; Takebayashi *et al.*, 2004). Ligand binding and subsequent EGFR phosphorylation has been shown to result in a migration of the EGFR out of caveolae/rafts (Mineo *et al.*, 1999; Abulrob *et al.*, 2004). Furthermore, a number of studies have demonstrated that disruption of lipid rafts (eg. with cholesterol-depleting agents such as methyl-β-cyclodextrin) leads to an increase in EGF binding and EGFR phosphorylation (Ushio-Fukai *et al.*, 2001; Chen and Resh, 2002; Pike and Casey, 2002; Ringerike *et al.*, 2002; Roepstorff *et al.*, 2002; Peres *et al.*, 2003; Westover *et al.*, 2003; Takebayashi *et al.*, 2004), suggesting that raft integrity is required for inhibition of EGFR activation. In the current study, n-3 PUFA treatment somewhat mimics the effect of cholesterol depletion in stimulating EGFR phosphorylation. We did not measure the effects of n-3 PUFA treatment on raft cholesterol levels in this study, as an earlier report suggested a lack of effect of n-3 PUFA (specifically EPA) on raft cholesterol levels in Jurkat T cells (Stulnig *et al.*, 2001). However, it is possible that n-3 PUFA modify the structure or function of lipid rafts by affecting raft cholesterol levels. In support, there is evidence that n-3 PUFA enrichment decreases the cholesterol content of both lipid rafts (Ma *et al.*, 2004a) and whole cell or model membranes (Hashimoto *et al.*, 1999; Brzustowicz *et al.*, 2002), perhaps due to steric incompatibility between cholesterol and long-chain n-3 PUFA (Brzustowicz *et al.*, 2002). Our data showing that n-3 PUFA (at least 60 μM EPA + 40 μM DHA) significantly decrease the content of raft SM support the hypothesis that n-3 PUFA modify the structure of lipid rafts, as sphingolipids are important for raft formation and function (Brown and London, 1998; Brown and London, 2000).

It is generally believed that increased activity of, or signaling through, the EGFR promotes cell proliferation and survival in most cell types. However, a number of recent reports have demonstrated increased EGFR phosphorylation concurrent with the induction of apoptosis by various agents (Reinehr *et al.*, 2003; Reinehr and Haussinger, 2004; Reinehr *et al.*, 2004; Im and Martinez, 2004). This has been observed in the MDA-MB-231 cell line (Cuadrado *et al.*, 2003). Sustained EGFR phosphorylation accompanying the induction of apoptosis has been associated with the activation of c-Jun NH(2)-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) (Reinehr *et al.*, 2003; Cuadrado *et al.*, 2003; Reinehr and Haussinger, 2004; Reinehr *et al.*, 2004; Tikhomirov and Carpenter, 2004). In addition, activated EGFR has also been shown to be required for activation of the CD95 death receptor system (Reinehr *et al.*, 2003; Reinehr and Haussinger, 2004; Reinehr *et al.*, 2004). It has been suggested that the outcome of EGFR signaling may depend on the concentration of EGFR/ErbB receptors in the cell, as mammary carcinoma cells expressing elevated levels of EGFR and ErbB-2 underwent apoptosis in response to EGF, whereas mammary tumor cells with low levels of the EGFR (and low or high levels of ErbB-2) survived and proliferated in the presence of EGF (Tikhomirov and Carpenter, 2004). Thus it appears that the EGFR may play a role in both cell growth and cell death (this has been the subject of a recent review by Danielsen and Maihle, 2002), with the outcome possibly dependent on cell-specific factors such as the level of EGFR expression.

The effects of n-3 PUFA on EGFR activation or signaling are not well established. In one report, EPA and DHA were the most effective PUFAs to increase tyrosine phosphorylation of the EGFR in human endothelial cells *in vitro* (Vacaresse *et al.*, 1999). In another, a mixture of EPA and DHA increased EGF-induced receptor tyrosine kinase activity (presumably EGFR activity) in EMT6 mouse mammary carcinoma cells *in vitro* (Estes *et al.*, 1997). Thus our data is in agreement with these two studies suggesting that n-3 PUFA treatment leads to an increase in EGFR phosphorylation and activation. The increased EGFR phosphorylation induced by n-3 PUFA in the current study does not appear to be associated with growth-promotion, as EPA and DHA significantly inhibited the growth of the MDA-MB-231 cells. Previous work outlined in chapter 3 demonstrates that EPA and DHA induce apoptotic cell death and decrease Akt Ser473 phosphorylation, as well as NFκB DNA-binding activity, in the MDA-MB-231 cell line. Thus, it appears that the increased EGFR phosphorylation observed with n-3 PUFA treatment in this study may be associated with signaling pathways leading to apoptosis, rather than cell survival (such as the Akt/NFkB cell survival pathway). Based on our findings, we can not rule out that the exclusion from rafts and subsequent activation of the EGFR is a side effect of n-3 PUFA-induced raft modification, and is not related to changes in mammary tumor cell growth observed with n-3 PUFA treatment. Further research is needed to clarify these observations.

The results in the current study suggest that EPA and DHA may also reduce the amount of PLCγ in lipid rafts of n-3 PUFA-treated MDA-MB-231 cells. Cholesterol depletion with methyl-β-cyclodextrin has been shown to inhibit raft localization of PLCγ (Wang *et al.*, 2001; Hur *et al.*, 2004), suggesting that raft integrity is also required for PLCγ raft localization. The activity of PLCγ following n-3 PUFA treatment was not assessed in the current research; however other reports have implicated a requirement for PLCγ localization in rafts for its activation. Phosphorylated PLCγ has been identified in rafts (Xavier *et al.*, 1998; Zhang *et al.*, 1998) and PLCγ recruitment to lipid rafts appears to be necessary for its tyrosine phosphorylation and activation (Veri *et al.*, 2001), whereas disruption of rafts decreases PLCγ phosphorylation or activation (Xavier *et al.*, 1998; Bodin *et al.*, 2003). There is some evidence that arachidonic acid plays a role in the activation of PLCγ (McGahon and Lynch, 1998; Sekiya *et al.*, 1999). Consistent with our fatty acid compositional data, n-3 PUFA may affect PLCγ raft localization and/or activity in MDA-MB-231 cells via lowering the concentration of AA in rafts and/or whole membrane phospholipids.

The results of this study suggest that raft localization of PI3K may be increased following tumor cell treatment with n-3 PUFA. The consequence of this observation is unknown. The p85 and p55 regulatory subunits of PI3K have been detected in rafts

(Xavier *et al.*, 1998; Zhang *et al.*, 1998), but relatively little is known about the role of rafts in PI3K activation. One report indicated that raft disruption by cholesterol depletion decreased raft localization of the p85 regulatory subunit of PI3K as well as its activation (as evidenced by a decrease in the formation of the two major PI3K lipid products, PIP_2 and PIP_3) in monkey kidney cells (Peres *et al.*, 2003). Such data suggests that PI3K activation occurs in rafts. However, data presented in chapter 3, showing that n-3 PUFA decrease Akt phosphorylation (which is regulated by PI3K activity) do not support PI3K activation by n-3 PUFA. It is possible that differences exist in the function and activation of the different PI3K regulatory subunits. In the research presented in this chapter, the antibody to the p85 regulatory subunit of PI3K recognized a band of ~ 55 kDa (in raft samples as well as whole cell lysates) in the MDA-MB-231 cell line, suggesting that the p55 regulatory subunit of PI3K predominates in this cell line. The p55 regulatory subunit is homologous to the COOH-terminal portion of p85, and lacks an amino-terminal SH3 domain, a proline-rich motif, and a bcr (breakpoint cluster region) homology domain found in the full-length p85 protein (Pons *et al.*, 1995; Vivanco and Sawyers, 2002). These missing regions may significantly alter the signaling potential of p55 (Pons *et al.*, 1995). Whether the different PI3K regulatory subunits are differentially modulated by raft localization is not known at this point. Further research is needed to clarify the role of rafts in PI3K activation and the potential effects of n-3 PUFA-induced increased PI3K in rafts on tumor cell growth and apoptosis.

There was no effect of n-3 PUFA treatment on the membrane distribution of Akt. Previous work (described in chapter 3) demonstrated that n-3 PUFA significantly decreased the phosphorylation (of Ser473) of Akt. These data suggest that n-3 PUFA do not alter the phosphorylation of Akt by altering its presence in rafts. However, it is possible that n-3 PUFA modulate raft localization of the kinases or phosphatases that are involved in the regulation of Akt phosphorylation. In support, Hill *et al.* (2002) recently identified an Akt Ser473 kinase activity enriched in membrane rafts from human embryonic kidney cells. It has been reported that raft disruption by cholesterol depletion strongly reduces Akt (Ser473) phosphorylation (Zhuang *et al.*, 2002; Peres *et al.*, 2003; Elhyany *et al.*, 2004), suggesting that raft integrity is important for Akt activation. Interestingly, in the study by Peres *et al.* (2003), cholesterol depletion decreased Akt phosphorylation and also increased EGFR phosphorylation, demonstrating that, similar to the findings presented in this research, decreased Akt phosphorylation was observed despite an increase in EGFR phosphorylation.

The results presented in this chapter demonstrate that n-3 PUFA have significant effects on the fatty acid composition and phospholipid content of lipid rafts in human breast cancer cells. These lipid alterations may modify raft structure or function, and significantly impact the location (EGFR, and possibly PLCγ and PI3K) and function (EGFR) of signaling molecules within the cell membrane. The data presented in this chapter describe a novel membrane mechanism by which n-3 PUFA may inhibit the growth of breast cancer cells.

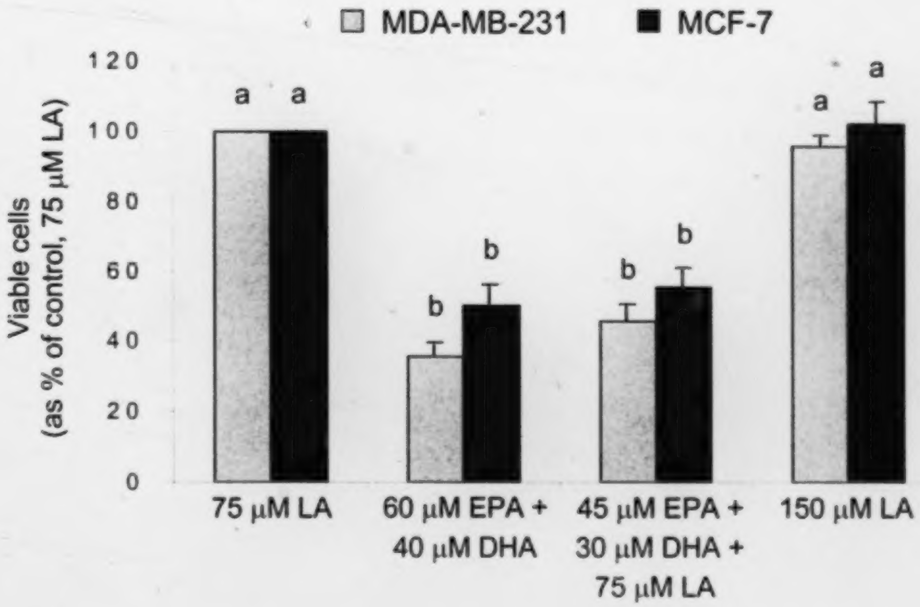

Figure 5-1 Effect of fatty acids on MDA-MB-231 and MCF-7 breast cancer cell growth. Viable cells were counted to determine cell population growth after 72 hours exposure to fatty acids, as described in "Materials and Methods". Within each cell line, values were normalized to a percentage of the control treatment (75 μM LA), which was taken as 100 %. Bars represent the means ± SE of ≥ 6 experiments. Within each cell line, bars that do not share a letter are significantly different ($p<0.05$).

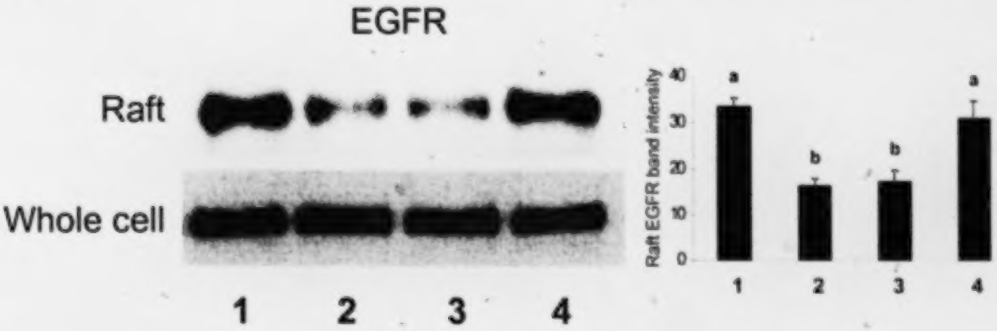

Figure 5-2 Effect of n-3 PUFA on lipid raft EGFR levels in MDA-MB-231 cells. Raft and whole cell protein lysates were prepared from MDA-MB-231 cells exposed to fatty acid treatment for 72 hours. Equal amounts of protein from each fatty acid treatment were immunoblotted for detection of the EGFR. Blots shown are representative of 4 independent experiments. The histogram depicts raft EGFR band intensities (means ± SE of 4 experiments) as determined by densitometric scanning of western blot autoradiograms. Bars that do not share a letter are significantly different ($p<0.05$). 1 = 75 μM LA; 2 = 60 μM EPA + 40 μM DHA; 3 = 45 μM EPA + 30 μM DHA + 75 μM LA; 4 = 150 μM LA.

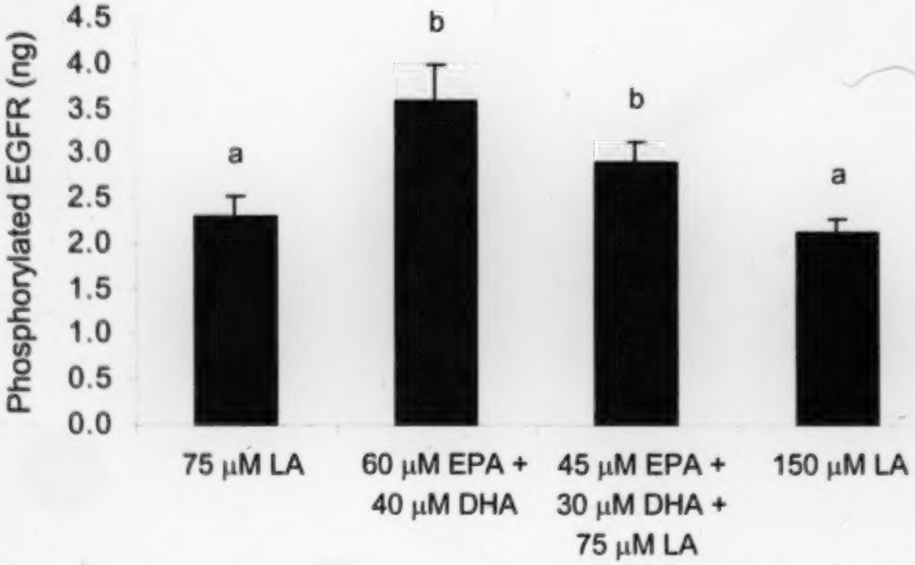

Figure 5-3 Effect of n-3 PUFA on whole cell EGFR phosphorylation levels in MDA-MB-231 cells. MDA-MB-231 cells were exposed to fatty acids for 72 hours. Cells were stimulated with 50 ng/mL EGF for 30 minutes at 37 °C, and then amounts of phosphorylated EGFR (ng) were determined as described in "Materials and Methods". Bars represent the means ± SE of 6 experiments. Values were log-transformed prior to statistical analysis. Bars that do not share a letter are significantly different ($p<0.05$).

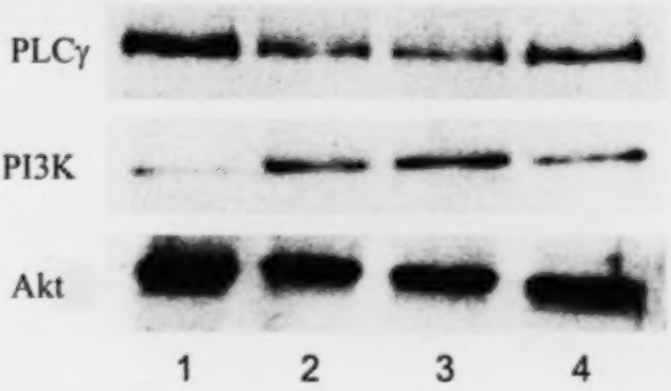

Figure 5-4 Effect of n-3 PUFA treatment on raft distribution of PLCγ, PI3K, and Akt in MDA-MB-231 cells. Protein lysates were prepared from lipid rafts of MDA-MB-231 cells exposed to fatty acid treatment for 72 hours. Equal amounts of protein from each fatty acid treatment were immunoblotted for detection of PLCγ, Akt and PI3K. Blots shown are representative of 2-3 independent experiments. 1 = 75 μM LA; 2 = 60 μM EPA + 40 μM DHA; 3 = 45 μM EPA + 30 μM DHA + 75 μM LA; 4 = 150 μM LA.

Table 5-1 Fatty acid composition of lipid rafts and whole cell membrane phospholipids of MDA-MB-231 breast cancer cells following incubation with polyunsaturated fatty acids[1]

	Lipid rafts				**Whole membrane PL**			
	75 µM LA	**60 µM EPA + 40 µM DHA**	**45 µM EPA + 30 µM DHA + 75 µM LA**	**150 µM LA**	**75 µM LA**	**60 µM EPA + 40 µM DHA**	**45 µM EPA + 30 µM DHA + 75 µM LA**	**150 µM LA**
Fatty acids	**% w/w of total fatty acids**				**% w/w of total fatty acids**			
14:0	1.84 ± 0.2^{ab}	2.43 ± 0.1^{a}	1.72 ± 0.2^{b}	1.61 ± 0.1^{b}	0.65 ± 0.0^{a}	1.33 ± 0.1^{b}	0.73 ± 0.1^{a}	0.64 ± 0.1^{a}
15:0	0.40 ± 0.0^{a}	0.56 ± 0.1^{b}	0.44 ± 0.1^{a}	0.37 ± 0.0^{a}	0.19 ± 0.0^{a}	0.30 ± 0.0^{b}	0.21 ± 0.0^{a}	0.19 ± 0.0^{a}
16:0	**35.62 ± 1.9^{a}**	**43.43 ± 1.3^{b}**	**32.72 ± 1.8^{a}**	**33.00 ± 1.8^{a}**	**14.80 ± 0.2^{a}**	**24.00 ± 0.3^{b}**	**15.37 ± 0.9^{a}**	**13.72 ± 0.4^{a}**
16:1 (n-7)	0.26 ± 0.0^{a}	0.42 ± 0.1^{b}	0.34 ± 0.1^{ab}	0.27 ± 0.0^{a}	0.36 ± 0.0^{a}	0.71 ± 0.1^{b}	0.51 ± 0.1^{c}	0.32 ± 0.0^{a}
17:0	1.35 ± 0.2	1.43 ± 0.1	1.41 ± 0.2	1.26 ± 0.2	0.79 ± 0.1	0.93 ± 0.0	0.75 ± 0.0	0.83 ± 0.2
17:1 (n-7)	0.87 ± 0.6	1.24 ± 0.6	1.56 ± 0.3	1.32 ± 0.2	1.80 ± 0.2^{a}	2.00 ± 0.2^{a}	1.75 ± 0.2^{ab}	1.50 ± 0.3^{b}
18:0	21.44 ± 0.9^{a}	17.57 ± 0.9^{b}	21.21 ± 2.5^{ab}	18.82 ± 0.6^{ab}	18.09 ± 0.4^{a}	18.34 ± 0.9^{a}	18.07 ± 0.4^{a}	16.46 ± 0.3^{b}
18:1 (n-7 + n-9)	7.59 ± 0.7	7.35 ± 0.6	8.55 ± 1.9	6.38 ± 0.3	9.64 ± 0.3^{a}	11.34 ± 0.5^{b}	8.89 ± 0.4^{a}	7.28 ± 0.3^{c}
18:2 (n-6)	**16.02 ± 0.7^{a}**	**0.76 ± 0.1^{b}**	**17.75 ± 2.2^{ac}**	**21.98 ± 0.3^{c}**	**33.13 ± 0.8^{a}**	**1.70 ± 0.1^{b}**	**33.12 ± 0.7^{a}**	**38.82 ± 1.2^{c}**
20:0	0.34 ± 0.0	0.40 ± 0.0	0.44 ± 0.1	0.31 ± 0.1	0.17 ± 0.0^{a}	0.28 ± 0.0^{b}	0.19 ± 0.0^{a}	0.15 ± 0.0^{a}
20:1 (n-7 + n-9)	0.21 ± 0.1	0.09 ± 0.1	0.32 ± 0.2	0.23 ± 0.1	0.33 ± 0.0^{a}	0.24 ± 0.0^{ab}	0.12 ± 0.1^{b}	0.27 ± 0.0^{ab}
20:2 (n-6)	4.20 ± 0.2^{a}	0.03 ± 0.0^{b}	1.14 ± 0.5^{b}	5.14 ± 0.4^{a}	7.02 ± 0.2^{a}	0.13 ± 0.1^{b}	3.15 ± 0.2^{c}	9.05 ± 0.3^{d}
20:3 (n-6)	0.94 ± 0.1^{a}	0.40 ± 0.0^{b}	0.39 ± 0.1^{b}	0.87 ± 0.0^{a}	1.58 ± 0.1^{a}	0.71 ± 0.0^{b}	0.64 ± 0.0^{b}	1.35 ± 0.1^{c}
20:4 (n-6)	**2.80 ± 0.5^{a}**	**1.36 ± 0.2^{b}**	**1.16 ± 0.2^{b}**	**2.25 ± 0.2^{a}**	**6.12 ± 0.3^{a}**	**3.32 ± 0.1^{b}**	**2.86 ± 0.1^{b}**	**4.52 ± 0.3^{c}**
20:5 (n-3)	**0.03 ± 0.0^{a}**	**7.36 ± 0.2^{b}**	**2.18 ± 0.5^{c}**	**0.05 ± 0.0^{a}**	**0.09 ± 0.0^{a}**	**15.83 ± 0.6^{b}**	**5.01 ± 0.5^{c}**	**0.13 ± 0.0^{a}**
22:0	0.71 ± 0.0^{a}	1.41 ± 0.1^{b}	0.92 ± 0.1^{a}	0.62 ± 0.1^{a}	0.29 ± 0.1^{ab}	0.41 ± 0.0^{a}	0.32 ± 0.1^{ab}	0.24 ± 0.0^{b}
22:2 (n-6)	0.54 ± 0.0^{a}	0.05 ± 0.1^{b}	0.06 ± 0.1^{b}	0.66 ± 0.1^{a}	0.45 ± 0.0^{a}	ND	0.06 ± 0.0^{b}	0.62 ± 0.0^{c}
22:4 (n-6)	0.72 ± 0.2^{a}	0.23 ± 0.0^{b}	0.21 ± 0.0^{b}	0.65 ± 0.1^{a}	1.46 ± 0.1^{a}	0.38 ± 0.0^{b}	0.30 ± 0.0^{b}	1.20 ± 0.1^{a}
22:5 (n-3)	**0.47 ± 0.1^{a}**	**4.54 ± 0.4^{b}**	**1.89 ± 0.6^{c}**	**0.44 ± 0.0^{a}**	**0.79 ± 0.1^{a}**	**8.65 ± 0.3^{b}**	**3.37 ± 0.1^{c}**	**0.69 ± 0.1^{a}**
24:0	1.56 ± 0.2	2.06 ± 0.3	2.08 ± 0.4	1.62 ± 0.5	0.54 ± 0.1	0.49 ± 0.0	0.46 ± 0.1	0.46 ± 0.1
22:6 (n-3)	**0.58 ± 0.1^{a}**	**5.17 ± 0.3^{b}**	**2.05 ± 0.5^{c}**	**0.55 ± 0.1^{a}**	**1.09 ± 0.1^{a}**	**8.40 ± 0.5^{b}**	**3.70 ± 0.3^{c}**	**0.98 ± 0.1^{a}**
24:1 (n-9)	1.08 ± 0.1	1.31 ± 0.0	1.01 ± 0.1	1.11 ± 0.2	0.48 ± 0.1	0.37 ± 0.1	0.34 ± 0.1	0.40 ± 0.1
Σ n-3 PUFA	1.37 ± 0.2^{a}	17.34 ± 0.5^{b}	6.43 ± 1.6^{c}	1.41 ± 0.0^{a}	1.99 ± 0.1^{a}	32.92 ± 1.2^{b}	12.09 ± 0.8^{c}	1.84 ± 0.2^{a}
Σ n-6 PUFA	25.23 ± 1.6^{ac}	2.82 ± 0.2^{b}	20.72 ± 2.9^{a}	31.55 ± 0.9^{c}	49.76 ± 0.4^{a}	6.25 ± 0.2^{b}	40.14 ± 0.5^{c}	55.56 ± 0.7^{d}
Σ SFA	63.32 ± 2.7^{ab}	69.35 ± 1.6^{b}	61.00 ± 3.3^{a}	57.68 ± 1.1^{a}	35.59 ± 0.5^{a}	46.15 ± 1.0^{b}	36.14 ± 0.9^{a}	32.79 ± 0.7^{c}
Σ MUFA	10.07 ± 1.1	10.49 ± 0.9	11.85 ± 1.5	9.36 ± 0.4	12.66 ± 0.2^{a}	14.69 ± 0.4^{b}	11.63 ± 0.3^{c}	9.80 ± 0.1^{d}
Σ PUFA	26.60 ± 1.8^{ab}	20.16 ± 0.7^{b}	27.15 ± 4.5^{ab}	32.96 ± 0.9^{a}	51.75 ± 0.5^{a}	39.17 ± 1.1^{b}	52.23 ± 1.1^{a}	57.41 ± 0.7^{c}
UI	0.75 ± 0.1^{a}	1.11 ± 0.0^{b}	0.90 ± 0.1^{ab}	0.86 ± 0.0^{ab}	1.40 ± 0.0^{a}	2.08 ± 0.1^{b}	1.63 ± 0.0^{c}	1.44 ± 0.0^{a}

[1] MDA-MB-231 breast cancer cells were exposed to fatty acids for 72 hours, followed by raft isolation. Fatty acid composition of lipid rafts and whole cell membrane phospholipids was determined from 3-4 experiments. Values are percentages of the total fatty acids and are expressed as means ± SE. Fatty acid data that were not normally distributed were log-transformed prior to statistical analysis. Values within a row (and within either rafts or whole cells) without a common superscript are significantly different ($p<0.05$). Values within a row with no superscripts are not significantly different. ND, not detectable; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UI, unsaturation index (calculated as the sum total of the number of double bonds per fatty acid multiplied by its mol %).

Table 5-2 Fatty acid composition of lipid rafts and whole cell membrane phospholipids of MCF-7 breast cancer cells following incubation with polyunsaturated fatty acids[1]

	Lipid rafts				**Whole membrane PL**			
	75 µM LA	**60 µM EPA + 40 µM DHA**	**45 µM EPA + 30 µM DHA + 75 µM LA**	**150 µM LA**	**75 µM LA**	**60 µM EPA + 40 µM DHA**	**45 µM EPA + 30 µM DHA + 75 µM LA**	**150 µM LA**
Fatty acids		**% w/w of total fatty acids**				**% w/w of total fatty acids**		
14:0	3.13 ± 0.2	5.11 ± 0.6	3.64 ± 0.6	3.81 ± 0.6	1.10 ± 0.1^{a}	1.91 ± 0.4^{b}	1.22 ± 0.2^{a}	0.99 ± 0.1^{a}
15:0	0.42 ± 0.0^{ab}	0.52 ± 0.0^{a}	0.41 ± 0.0^{b}	0.37 ± 0.0^{b}	0.18 ± 0.0^{ac}	0.29 ± 0.0^{b}	0.21 ± 0.0^{a}	0.16 ± 0.0^{c}
16:0	$\mathbf{38.12 \pm 0.6^{a}}$	$\mathbf{45.75 \pm 2.0^{b}}$	$\mathbf{35.55 \pm 2.3^{a}}$	$\mathbf{36.56 \pm 0.6^{a}}$	$\mathbf{17.72 \pm 0.5^{ac}}$	$\mathbf{26.79 \pm 1.0^{b}}$	$\mathbf{19.45 \pm 0.4^{a}}$	$\mathbf{16.95 \pm 0.4^{c}}$
16:1 (n-7)	0.70 ± 0.0^{ac}	1.37 ± 0.1^{b}	0.92 ± 0.0^{a}	0.60 ± 0.1^{c}	1.02 ± 0.1^{a}	1.99 ± 0.1^{b}	1.11 ± 0.1^{a}	0.71 ± 0.0^{c}
17:0	0.73 ± 0.1	0.70 ± 0.1	0.66 ± 0.1	0.70 ± 0.0	0.46 ± 0.0	0.68 ± 0.1	0.52 ± 0.0	0.56 ± 0.1
17:1 (n-7)	0.16 ± 0.1	0.15 ± 0.1	0.14 ± 0.0	0.15 ± 0.0	0.28 ± 0.0^{a}	0.39 ± 0.0^{b}	0.22 ± 0.0^{ac}	0.18 ± 0.0^{c}
18:0	19.40 ± 0.7^{ab}	18.49 ± 0.8^{a}	19.97 ± 0.3^{b}	20.18 ± 0.4^{b}	15.50 ± 0.8^{a}	20.98 ± 0.4^{b}	18.38 ± 0.2^{c}	17.09 ± 0.5^{ac}
18:1 (n-7 + n-9)	9.05 ± 0.5^{ab}	10.58 ± 1.1^{a}	7.50 ± 0.5^{b}	6.84 ± 0.3^{b}	11.92 ± 0.6^{a}	13.91 ± 0.4^{b}	8.73 ± 0.3^{c}	7.15 ± 0.2^{d}
18:2 (n-6)	$\mathbf{18.76 \pm 0.6^{a}}$	$\mathbf{0.66 \pm 0.0^{b}}$	$\mathbf{20.37 \pm 1.8^{a}}$	$\mathbf{22.86 \pm 0.1^{a}}$	$\mathbf{39.33 \pm 0.3^{a}}$	$\mathbf{1.82 \pm 0.1^{b}}$	$\mathbf{38.45 \pm 0.8^{a}}$	$\mathbf{46.63 \pm 0.7^{c}}$
20:0	0.59 ± 0.2	0.39 ± 0.0	0.39 ± 0.1	0.54 ± 0.2	0.21 ± 0.0^{a}	0.27 ± 0.0^{ab}	0.27 ± 0.0^{b}	0.26 ± 0.0^{ab}
20:1 (n-7 + n-9)	0.20 ± 0.1	0.08 ± 0.0	0.11 ± 0.0	0.10 ± 0.1	0.49 ± 0.0^{a}	0.18 ± 0.0^{b}	0.16 ± 0.0^{b}	0.24 ± 0.0^{b}
20:2 (n-6)	2.48 ± 0.2^{a}	0.12 ± 0.1^{b}	0.76 ± 0.1^{b}	2.63 ± 0.1^{a}	6.45 ± 0.5^{a}	0.11 ± 0.0^{b}	1.61 ± 0.1^{c}	5.70 ± 0.7^{a}
20:3 (n-6)	0.07 ± 0.0	0.16 ± 0.1	0.13 ± 0.0	0.04 ± 0.0	0.20 ± 0.0^{ac}	0.52 ± 0.0^{b}	0.26 ± 0.0^{a}	0.14 ± 0.0^{c}
20:4 (n-6)	$\mathbf{1.04 \pm 0.0^{a}}$	$\mathbf{0.84 \pm 0.1^{ab}}$	$\mathbf{0.59 \pm 0.1^{bc}}$	$\mathbf{0.53 \pm 0.0^{c}}$	$\mathbf{2.02 \pm 0.2^{a}}$	$\mathbf{1.97 \pm 0.1^{a}}$	$\mathbf{1.01 \pm 0.0^{b}}$	$\mathbf{0.82 \pm 0.0^{b}}$
20:5 (n-3)	$\mathbf{0.01 \pm 0.0^{a}}$	$\mathbf{6.21 \pm 1.1^{b}}$	$\mathbf{1.86 \pm 0.3^{b}}$	$\mathbf{0.06 \pm 0.0^{a}}$	$\mathbf{0.18 \pm 0.1^{a}}$	$\mathbf{17.71 \pm 0.9^{b}}$	$\mathbf{3.76 \pm 0.2^{c}}$	$\mathbf{0.06 \pm 0.0^{a}}$
22:0	0.92 ± 0.1	1.06 ± 0.1	1.00 ± 0.2	0.67 ± 0.1	0.24 ± 0.0	0.29 ± 0.0	0.29 ± 0.0	0.25 ± 0.0
22:2 (n-6)	0.39 ± 0.0^{a}	ND	0.13 ± 0.0^{b}	0.40 ± 0.1^{a}	0.52 ± 0.0^{a}	ND	0.03 ± 0.0^{b}	0.46 ± 0.1^{a}
22:4 (n-6)	0.19 ± 0.1	ND	0.07 ± 0.1	0.09 ± 0.1	0.31 ± 0.0^{a}	0.11 ± 0.0^{b}	0.02 ± 0.0^{c}	0.17 ± 0.0^{d}
22:5 (n-3)	$\mathbf{0.33 \pm 0.1^{a}}$	$\mathbf{2.02 \pm 0.3^{b}}$	$\mathbf{0.88 \pm 0.1^{c}}$	$\mathbf{0.18 \pm 0.0^{d}}$	$\mathbf{0.45 \pm 0.0^{a}}$	$\mathbf{4.25 \pm 0.4^{b}}$	$\mathbf{1.41 \pm 0.1^{c}}$	$\mathbf{0.34 \pm 0.0^{a}}$
24:0	0.82 ± 0.1	1.01 ± 0.1	1.24 ± 0.3	0.57 ± 0.2	0.20 ± 0.0	0.21 ± 0.0	0.22 ± 0.0	0.20 ± 0.0
22:6 (n-3)	$\mathbf{0.38 \pm 0.0^{a}}$	$\mathbf{2.55 \pm 0.4^{b}}$	$\mathbf{1.10 \pm 0.2^{c}}$	$\mathbf{0.18 \pm 0.0^{d}}$	$\mathbf{0.60 \pm 0.1^{a}}$	$\mathbf{5.11 \pm 0.2^{b}}$	$\mathbf{2.03 \pm 0.2^{c}}$	$\mathbf{0.31 \pm 0.0^{a}}$
24:1 (n-9)	1.33 ± 0.1	1.59 ± 0.4	1.91 ± 0.4	0.96 ± 0.1	0.44 ± 0.1^{a}	0.28 ± 0.1^{b}	0.53 ± 0.1^{a}	0.44 ± 0.1^{a}
Σ n-3 PUFA	1.26 ± 0.1^{a}	11.35 ± 1.4^{b}	4.32 ± 0.4^{c}	1.18 ± 0.2^{a}	1.27 ± 0.0^{a}	27.19 ± 1.4^{b}	7.21 ± 0.2^{c}	0.74 ± 0.1^{d}
Σ n-6 PUFA	22.95 ± 0.7^{a}	1.78 ± 0.1^{b}	22.05 ± 2.0^{a}	26.55 ± 0.2^{a}	48.82 ± 0.5^{a}	4.53 ± 0.2^{b}	41.38 ± 0.7^{c}	53.91 ± 0.4^{d}
Σ SFA	64.19 ± 1.2^{a}	73.05 ± 3.1^{b}	62.88 ± 3.2^{a}	63.47 ± 0.3^{a}	35.64 ± 0.4^{a}	51.48 ± 1.1^{b}	40.65 ± 0.4^{c}	36.55 ± 0.7^{a}
Σ MUFA	11.60 ± 0.4^{a}	13.83 ± 1.7^{a}	10.74 ± 0.7^{ab}	8.80 ± 0.2^{b}	14.26 ± 0.7^{a}	16.80 ± 0.5^{b}	10.77 ± 0.3^{c}	8.80 ± 0.3^{d}
Σ PUFA	24.21 ± 0.8^{a}	13.13 ± 1.5^{b}	26.37 ± 2.5^{a}	27.73 ± 0.2^{a}	50.09 ± 0.5^{a}	31.72 ± 1.5^{b}	48.59 ± 0.6^{a}	54.65 ± 0.5^{c}
UI	0.66 ± 0.0	0.77 ± 0.1	0.78 ± 0.1	0.68 ± 0.0	1.24 ± 0.0^{a}	1.71 ± 0.1^{b}	1.34 ± 0.0^{a}	1.23 ± 0.0^{a}

[1] MCF-7 breast cancer cells were exposed to fatty acids for 72 hours, followed by raft isolation. Fatty acid composition of lipid rafts and whole cell membrane phospholipids was determined from 3-4 experiments. Values are percentages of the total fatty acids and are expressed as means ± SE. Fatty acid data that were not normally distributed were log-transformed prior to statistical analysis. Values within a row (and within either rafts or whole cells) without a common superscript are significantly different ($p<0.05$). Values within a row with no superscripts are not significantly different. ND, not detectable; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UI, unsaturation index (calculated as the sum total of the number of double bonds per fatty acid multiplied by its mol %).

Table 5-3 Effect of n-3 PUFA on phospholipid composition of lipid rafts from human breast cancer cells

	Phospholipid class				
Fatty acid treatment	**PE**	**PI**	**PS**	**PC**	**SM**
	μg/100 μg total phospholipid phosphorus				
MDA-MB-231:					
75 μM LA	14.61 ± 0.7^{ab}	3.82 ± 0.2^{ab}	7.31 ± 0.1	49.22 ± 0.8^{a}	25.04 ± 0.6^{ac}
60 μM EPA + 40 μM DHA	12.96 ± 0.7^{a}	3.36 ± 0.1^{b}	7.71 ± 0.8	57.00 ± 0.7^{b}	18.97 ± 1.0^{b}
45 μM EPA + 30 μM DHA + 75 μM LA	16.31 ± 0.2^{b}	4.16 ± 0.1^{ab}	7.51 ± 0.3	49.62 ± 1.2^{a}	22.40 ± 1.2^{c}
150 μM LA	14.81 ± 0.5^{ab}	4.44 ± 0.4^{a}	7.57 ± 0.2	46.68 ± 1.3^{a}	26.51 ± 1.2^{a}
MCF-7:					
75 μM LA	14.61 ± 0.5^{ab}	6.32 ± 0.7	2.73 ± 1.0	51.82 ± 1.1^{a}	24.53 ± 0.9^{a}
60 μM EPA + 40 μM DHA	13.35 ± 0.2^{a}	7.84 ± 0.6	2.74 ± 1.0	59.10 ± 2.5^{b}	16.98 ± 1.0^{b}
45 μM EPA + 30 μM DHA + 75 μM LA	17.73 ± 0.5^{b}	7.59 ± 0.4	2.91 ± 1.2	51.05 ± 0.4^{a}	20.72 ± 1.4^{ab}
150 μM LA	16.32 ± 1.7^{ab}	6.57 ± 0.8	2.51 ± 0.9	49.80 ± 2.7^{a}	24.80 ± 1.7^{a}

Values are the percentage of the total phospholipid pool represented by each phospholipid class, and are the mean ± SE of 3 to 6 independent experiments. Values within a column, and within each cell line, that do not share a letter are significantly different ($p<0.05$). PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

Table 5-4 Effect of n-3 PUFA on whole cell ceramide levels in MDA-MB-231 cells

Fatty acid treatment	pmol ceramide/nmol phosphorus
75 μM LA	7.12 ± 0.8
60 μM EPA + 40 μM DHA	8.06 ± 1.2
45 μM EPA + 30 μM DHA + 75 μM LA	7.63 ± 0.2
150 μM LA	8.44 ± 0.3

Values are the average ± SD of 2 experiments.

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6. GENERAL SUMMARY AND DISCUSSION

6.1 SUMMARY OF RESULTS

The objectives of this research were to test the following hypotheses:

I. Long-chain n-3 fatty acids inhibit mammary tumor growth *in vitro* through a reduction in cellular proliferation, an increase in apoptosis, or a combination of both.

This hypothesis was supported by the results reported in chapter 3. Specifically, it was hypothesized that:

A. A combination of EPA and DHA will reduce cellular proliferation ([methyl-^{3}H]-thymidine incorporation, expression of proliferation-related and cell cycle regulatory proteins) of mammary tumor cells relative to the control.

The results reported in chapter 3 support this hypothesis. A combination of EPA and DHA significantly reduced [methyl-^{3}H]-thymidine incorporation and expression of the proliferation-associated proteins, PCNA and PRK, in both MDA-MB-231 and MCF-7 human breast cancer cells. The proportions of cyclin D1- and cyclin B1-positive cells were decreased in MDA-MB-231 cells with n-3 PUFA treatment, but the effect was not statistically significant.

B. A combination of EPA and DHA will induce apoptotic cell death of mammary tumor cells relative to the control.

The experiments described in chapter 3 provide evidence for this hypothesis. A combination of EPA and DHA significantly increased: i) the proportion of cells with a loss of mitochondrial membrane potential, ii) the proportion of cells with activated caspase proteins (in MDA-MB-231 cells only), and iii) the extent of DNA fragmentation, all markers of apoptosis, in MDA-MB-231 and MCF-7 human breast cancer cells. Mammary tumor cells incubated with EPA and DHA did not significantly exhibit properties of necrosis (lactate dehydrogenase release, propidium iodide uptake), therefore cell death was believed to occur primarily via apoptosis.

C. The effects of EPA and DHA on mammary tumor cells are not due to a deficiency of LA in the cell culture medium and are still achieved when EPA and DHA are provided in combination with LA.

The results reported in chapter 3 support this hypothesis. A mixture of EPA and DHA in combination with LA significantly decreased mammary tumor cell growth (i.e. the number of viable cell counts) as well as measures of proliferation ([methyl-^{3}H]-thymidine incorporation, expression of proliferation-associated proteins), and increased measures of apoptosis (loss of mitochondrial membrane potential, caspase protein activity, DNA fragmentation) in both MDA-MB-231 and MCF-7 cells.

D. The effects of EPA and DHA on mammary tumor cell growth are not related to increased lipid peroxidation.

The results reported in chapter 3 support this hypothesis. The addition of dl-α-tocopherol (vitamin E) to the culture medium did not abrogate the growth-inhibitory effects of EPA or DHA on MDA-MB-231 cells.

II. Long-chain n-3 fatty acids are incorporated into mammary tumor cell membrane phospholipids and alter the function of cell survival signaling pathways.

This hypothesis was supported by the results reported in chapter 3. Specifically, it was hypothesized that:

E. EPA and DHA will be incorporated into cell membrane phospholipids of mammary tumor cells.

This hypothesis is supported by the results reported in chapter 3. In both MDA-MB-231 and MCF-7 cells, EPA and DHA were significantly incorporated into membrane phospholipids following incubation with EPA and DHA both in the absence and presence of LA. In both cell lines, incorporation of EPA and DHA was significantly lower when these PUFA were provided in the presence of LA versus in the absence of LA. Enrichment of n-3 PUFA in membrane phospholipids did not occur at the expense of LA in either cell line. However, n-3 PUFA treatment did significantly decrease AA concentrations (in PC, PE, PI and PS) in MDA-MB-231 cells both in the absence and presence of LA. In MCF-7 cells, AA concentrations were significantly reduced by n-3 PUFA in the presence and absence of LA only in PI; among the other phospholipids, AA concentrations were reduced primarily by 45 μM EPA + 30 μM DHA + 75 μM LA and by 150 μM LA.

F. A combination of EPA and DHA will decrease Akt phosphorylation in mammary tumor cells relative to the control.

The results reported in chapter 3 support this hypothesis. Both MDA-MB-231 and MCF-7 cells incubated with EPA and DHA exhibited decreased Akt (Ser473) phosphorylation relative to control-treated cells.

G. A combination of EPA and DHA will decrease NFκB activity in mammary tumor cells relative to the control.

The results reported in chapter 3 support this hypothesis in the MDA-MB-231 cell line. A combination of EPA and DHA decreased NFκB DNA-binding activity relative to control-treated cells. A decrease in NFκB DNA-binding activity could not be detected with n-3 PUFA treatment in MCF-7 cells.

III. Long-chain n-3 fatty acids are incorporated into mammary tumor cell membrane lipid rafts, and alter raft lipid and signaling protein composition, leading to functionally relevant changes in signal transduction.

A microscale method for the isolation of lipid rafts from mammary tumor cells was developed and described in chapter 4. The results obtained through the use of this microscale raft isolation method, as outlined in chapter 5, support the above hypothesis. Specifically, it was hypothesized that:

H. EPA and DHA will be incorporated into membrane lipid rafts of mammary tumor cells.

This hypothesis was supported by the results reported in chapter 5. In both MDA-MB-231 and MCF-7 cells, EPA and DHA were significantly incorporated into membrane lipid rafts when provided both in the presence and absence of LA. Enrichment of n-3 PUFA in lipid rafts did not occur at the expense of LA in either cell line. However, n-3 PUFA treatment did significantly decrease AA concentrations in lipid rafts of MDA-MB-231 cells both in the absence and presence of LA.

I. A combination of EPA and DHA will alter the mass distribution of phospholipids within lipid rafts of mammary tumor cells.

The results presented in chapter 5 support this hypothesis. EPA and DHA (in the absence of LA) significantly reduced the proportion (i.e. percentage of the total phospholipids) of sphingomyelin, and significantly increased the proportion of phosphatidylcholine, in lipid rafts isolated from n-3 PUFA-treated MDA-MB-231 and MCF-7 cells.

J. A combination of EPA and DHA will displace signaling proteins involved in tumor growth from lipid rafts, which will decrease their activities.

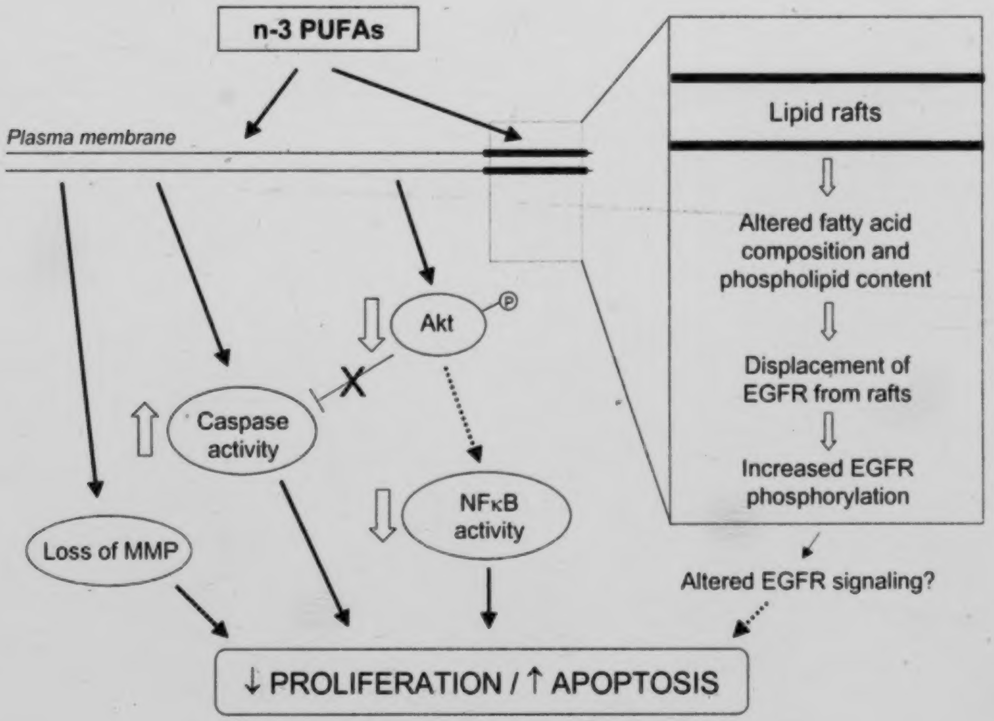
This hypothesis was partially supported by the results presented in chapter 5. A combination of EPA and DHA significantly reduced the protein levels of the epidermal growth factor receptor (EGFR), and possibly reduced protein levels of PLCγ as well, from lipid rafts of MDA-MB-231 breast cancer cells. The displacement of the EGFR from lipid rafts by n-3 PUFA correlated with an increase in EGFR phosphorylation. PLCγ activation status following n-3 PUFA treatment was not determined, so the impact of possible PLCγ displacement from lipid rafts by n-3 PUFA is not known. N-3 PUFA treatment did not appear to affect the localization of Akt in lipid rafts of MDA-MB-231 cells, despite the observed decrease in whole-cell Akt phosphorylation levels with n-3 PUFA treatment in these cells (as described in chapter 3). N-3 PUFA treatment may have increased protein levels of the p55 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) in lipid rafts of MDA-MB-231 cells. The implication of this increased raft localization of PI3K on its activation was not determined.

6.2 GENERAL DISCUSSION

The central issue addressed in this research was identification of mechanisms for reduced growth of mammary tumor cells induced by long-chain n-3 polyunsaturated fatty acids. Although previous studies have examined potential mechanisms for n-3 PUFA-induced tumor growth inhibition, there is little consensus among the published reports as to how n-3 PUFA decrease mammary tumor growth. Furthermore, it is not well established whether n-3 PUFA affected primarily tumor cell proliferation or cell death, or both. The results of the current research suggest that n-3 PUFA impact both tumor cell proliferation as well as cell death via apoptosis. These findings were observed in two human breast cancer cell lines (MDA-MB-231 and MCF-7) that differ in their growth characteristics and representative disease stage (Dickson *et al.*, 1986; Timoshenko *et al.*, 2003). The fact that the growth of both of these two phenotypically dissimilar cell lines is inhibited by n-3 PUFA suggests that the effects of n-3 PUFA are not limited to one particular cell line but may be relevant to a broader spectrum of mammary tumors.

An effect of n-3 PUFA on cell proliferation and/or death was important to establish, in order to move to the next step to focus on signal transduction pathways altered by n-3 PUFA treatment. The results presented in this research suggest that n-3 PUFA might influence both cell proliferation and survival pathways, as well as death-inducing pathways. The current research focused on the impact of n-3 PUFA on cell proliferation and survival signaling pathways.

The combined results of this thesis research support a mechanism for n-3 PUFA-induced mammary tumor growth inhibition that could be linked to n-3 PUFA enrichment in tumor cell membranes. Figure 6-1 is a schematic representation of the mechanisms by which n-3 PUFA appear to inhibit mammary tumor cell growth, particularly in the MDA-MB-231 cell line. The proposed mechanisms, however, do not exclude the possibility that other mechanisms (eg. eicosanoid production, gene expression) are also involved in n-3 PUFA-mediated growth suppression of mammary tumor cells.



n-3 PUFAs
Plasma membrane
Lipid rafts
Altered fatty acid composition and phospholipid content
Displacement of EGFR from rafts
Increased EGFR phosphorylation
Akt
P
Caspase activity
NFκB activity
Loss of MMP
Altered EGFR signaling?
↓ PROLIFERATION / ↑ APOPTOSIS

Figure 6-1 Hypothesized mechanisms of n-3 PUFA-induced growth inhibition of MDA-MB-231 cells. Dotted arrows indicate steps in the pathway have been omitted.

It is well-established that n-3 PUFA are incorporated into other tumor cell membrane phospholipids when provided *in vitro* and *in vivo* (Jurkowski and Cave, 1985; Karmali *et al.*, 1989; Grammatikos *et al.*, 1994; Hatala *et al.*, 1994; Rose *et al.*, 1995; Bardon *et al.*, 1996; Robinson *et al.*, 2002), and this research confirmed that EPA and DHA were significantly incorporated into cell membrane phospholipids of both MDA-MB-231 and MCF-7 cells incubated with n-3 PUFA with and without LA (chapter 3). AA concentrations were significantly reduced (in PC, PE, PI and PS) by n-3 PUFA treatment with or without LA in the MDA-MB-231 cell line. This is consistent with other reports demonstrating reduced AA concentrations in phospholipids enriched in n-3 PUFA (Jurkowski and Cave, 1985; Rose *et al.*, 1995; Robinson *et al.*, 2002). However, in MCF-7 cells, significant decreases in AA concentrations were observed primarily with the 150 μM LA and 45 μM EPA + 30 μM DHA + 75 μM LA treatments. This observation would suggest that the growth-inhibitory effects of n-3 PUFA on mammary tumor cells may be related more to the increased enrichment of n-3 PUFA in membrane phospholipids rather than to a decrease in membrane AA levels, at least in the MCF-7 cell line.

The concentrations of n-3 PUFA in mammary tumor cell phospholipids following treatment with 45 μM EPA + 30 μM DHA + 75 μM LA observed in this study were consistent with n-3 PUFA concentrations in phospholipids in a mammary tumor (R3230AC) of rats fed a diet containing 5 % w/w n-3 PUFA (Robinson *et al.*, 2002). For example, total n-3 PUFA concentrations in PE ranged from 9.9 to 23 % of the total fatty acids in MCF-7 and MDA-MB-231 cells, respectively, whereas total n-3 PUFA made up 14 % of the total fatty acids in PE of R3230AC mammary tumor cells from rats fed a diet containing 5 % w/w n-3 PUFA. DHA itself comprised 1.8 to 8.7 % of the total fatty acids in PE in MCF-7 and MDA-MB-231 cells respectively, compared to 6.8 % of the total fatty acids in PE from the R3230AC mammary tumor *in vivo* (Robinson *et al.*, 2002). Thus the enrichment levels of n-3 PUFA in mammary tumor cells incubated with EPA and DHA plus LA *in vitro* observed in this thesis research are consistent with those that are attainable through dietary manipulation (albeit with high doses of supplementary fatty acids), supporting the relevance of this research to a dietary model of mammary tumor growth inhibition.

Based on these findings, it was hypothesized that the inhibitory effects of n-3 PUFA on mammary tumor growth may be mediated through their incorporation into plasma membrane phospholipids and consequent effects on membrane-associated signaling events.

The Akt kinase plays a central role in cell proliferation and survival (Vivanco and Sawyers, 2002). Akt is activated by growth-stimulatory signals such as growth factors, hormones, and cytokines, and functions to promote cell survival and protect cells from apoptotic cell death by phosphorylating and inactivating components of the cell death machinery (eg. caspase-9, BAD), as well as activating prosurvival transcription factors such as NFκB (Vivanco and Sawyers, 2002). The Akt kinase must be recruited to the membrane to become activated (Vivanco and Sawyers, 2002), thus we hypothesized that membrane alterations induced by n-3 PUFA may impact on Akt activation. In support, our data suggests that in both MDA-MB-231 and MCF-7 cells, n-3 PUFA treatment decreases Akt phosphorylation. In addition, NFκB DNA-binding activity was also decreased by n-3 PUFA treatment (at least in MDA-MB-231 cells), which is consistent with the decreased activity of its upstream regulator, Akt. Inhibition of NFκB activity has been shown to render tumor cells susceptible to apoptosis (Sovak *et al.*, 1997), therefore the decreases in Akt phosphorylation and NFκB activity induced by n-3 PUFA observed in this thesis research represent a novel mechanism by which n-3 PUFA decrease the growth and induce apoptosis in breast cancer cells.

Alterations in NFκB DNA-binding activity could not be detected in MCF-7 cells. However, it has been suggested that the role of NFκB in promoting cell growth and survival may not be as important in estrogen receptor-positive cells, such as MCF-7, as these cells depend more on estrogen for growth stimulation than on endogenous NFκB signaling (Pratt *et al.*, 2003). In addition, the Akt kinase impacts on a multitude of signaling molecules other than NFκB, and so it is likely that other factors affected by Akt (eg. caspase-9, BAD, glycogen synthase kinase-3β, forkhead family of transcription factors; Datta *et al.*, 1999) may also affect proliferation and apoptosis in the MCF-7 cell line. Interestingly, Akt was shown to activate ERα in the absence of estrogen (Campbell *et al.*, 2001); thus Akt inhibition via n-3 PUFA may be important to decreasing ER stimulation of growth in these cells. Although decreased Akt activation and subsequent NFκB activation induced by n-3 PUFA represent a plausible mechanism by which n-3 PUFA may decrease the survival of mammary tumor cells, it must be considered that other mechanisms may be in operation concurrently, at least in estrogen receptor-positive breast cancer cells.

To further explore how changes in membrane fatty acid composition induced by n-3 PUFA could relate to alterations in membrane-associated signal transduction, we hypothesized that n-3 PUFA may be incorporated into membrane lipid rafts. It is generally accepted that these membrane microdomains within the plasma membrane play a key role in signal transduction (Simons and Toomre, 2000). The results of this research indicate that EPA and DHA were significantly incorporated into, and altered the phospholipid content, of membrane rafts of both MDA-MB-231 and MCF-7 cells incubated with n-3 PUFA. These results suggest that n-3 PUFA may dramatically alter the structure or properties of lipid rafts. In support, EPA and DHA induced changes in the distribution of signaling molecules within lipid rafts in MDA-MB-231 cells, displacing the EGFR, and potentially PLCγ, from lipid rafts, and potentially increasing p55 PI3K protein levels. The displacement of the EGFR from rafts correlated with increased EGFR phosphorylation, suggesting that the n-3 PUFA-induced alteration in EGFR membrane localization can affect its function. While raft localization of the Akt kinase was not disrupted by EPA and DHA treatment, phosphorylated Akt levels (in whole cells) were significantly reduced by n-3 PUFA, suggesting that raft localization of the kinases and phosphatases that govern Akt phosphorylation could be altered by n-3 PUFA. Further research is needed to confirm the effects of n-3 PUFA on the raft localization of PLCγ and PI3K, and how this may affect the activities of these proteins in relation to tumor cell growth and apoptosis.

Signaling through the EGFR has generally been thought to promote cell proliferation. However, the reduced levels of phosphorylated Akt seen with n-3 PUFA treatment do not indicate that the EGFR, in this case, is stimulating cell survival, at least through Akt. In fact, a number of studies have reported that, in some circumstances, signaling through the EGFR is associated with the induction of apoptosis (Reinehr *et al.*, 2003; Im and Martinez, 2004; Reinehr and Haussinger, 2004; Reinehr *et al.*, 2004). The findings in this thesis research suggest that n-3 PUFA treatment alters EGFR signaling in such a way as to favor the induction of apoptosis.

The effects of n-3 PUFA on signaling protein distribution in membrane rafts were not examined in the MCF-7 cell line. However, EPA and DHA were similarly incorporated into rafts from both MDA-MB-231 and MCF-7 cell lines, and in both cell lines, EPA and DHA induced similar changes in the proportions of raft sphingomyelin and phosphatidylcholine. Thus it is likely that the effects of EPA and DHA on raft lipid composition would also impact on signaling protein distribution in MCF-7 cells. The impact of n-3 PUFA on Akt phosphorylation warrants further investigation of n-3 PUFA-mediated raft modulation of proteins and lipids involved in Akt activation. In addition, several studies indicate that the MCF-7 cell line is growth-stimulated by insulin and the insulin-like growth factors (IGFs) (Karey and Sirbasku, 1988; Thorsen *et al.*, 1992; Levenson and Jordan, 1997). Lipid rafts appear to play a role in signaling through both the insulin and insulin-like growth factor receptors (Bickel, 2002; Vainio *et al.*, 2002; Huo *et al.*, 2003). Furthermore, a number of studies suggest that n-3 PUFA, provided either in the diet or in culture, can modulate aspects of insulin signaling in various cell types (Yorek *et al.*, 1989; Liu *et al.*, 1994; Murata *et al.*, 2001; Taouis *et al.*, 2002). Therefore examination of the effects of n-3 PUFA on raft modulation of insulin receptor and insulin-like growth factor receptor-associated signaling would be a logical next step.

In some experiments conducted in this thesis research, there were small differences between the effects of the 60 μM EPA + 40 μM DHA versus 45 μM EPA + 30 μM DHA + 75 μM LA treatments, suggesting a potential dose effect of n-3 PUFA on some tumor cell parameters. This is not unlikely considering the observation that concentrations of EPA and DHA were significantly lower in membrane phospholipids of cells incubated with EPA and DHA plus LA as compared to cells incubated with EPA and DHA alone. However, growth inhibition (i.e. a reduction in viable cell counts) occurred to a similar extent in both cell lines with both n-3 PUFA treatments, suggesting that there may be a minimum concentration of n-3 PUFA in membrane phospholipids required to alter tumor cell functions and ultimately inhibit tumor growth. Alternatively, it is possible that enrichment of n-3 PUFA in tumor cell membranes may be a side effect of n-3 PUFA treatment, and is not the root cause of n-3 PUFA-induced tumor cell growth inhibition. Further research is necessary to verify whether a dose-response relationship exists between increasing levels of n-3 PUFA enrichment in tumor cell membranes and decreased tumor cell growth/increased tumor cell death.

The research conducted in this thesis examined primarily signaling pathways/molecules involved in cell growth and survival. Future studies should also examine the effects of n-3 PUFA on apoptosis-inducing signals. Two major pathways of apoptosis induction exist in mammalian cells: the death receptor pathway and the mitochondrial pathway (sometimes referred to as the extrinsic and intrinsic pathways of apoptosis induction, respectively) (Hengartner, 2000; Okada and Mak, 2004). The death receptor pathway is triggered by members of the death receptor superfamily, mainly CD95/Fas and the tumor necrosis factor receptor (TNFR). Activation of these death receptors leads to receptor clustering and formation of a death-inducing signaling complex. This complex recruits multiple procaspase-8 molecules, resulting in caspase-8 activation (Hengartner, 2000). Effector caspases (eg. caspase-3, -6 and –7) are subsequently activated through a cascade of caspase activation. The mitochondrial pathway is triggered by various extracellular and intracellular stresses (eg. growth-factor withdrawal, hypoxia, DNA damage) (Okada and Mak, 2004). A series of biochemical events is induced that results in the permeabilization of the outer mitochondrial membrane and release of proapoptotic molecules such as cytochrome c (Okada and Mak, 2004). This leads to formation of the apoptosome – a large protein complex containing cytochrome c and caspase-9, which triggers activation of the effector caspases (Okada and Mak, 2004).

The Fas death receptor primarily induces programmed cell death when activated. TNFR, however, can exert different effects on cells ranging from cellular activation via NFκB activation to the induction of apoptosis (Muppidi *et al.*, 2004). The outcome of TNFR signaling may depend on the location of the receptor within lipid rafts (Muppidi *et al.*, 2004), as TNFR translocation into lipid rafts has been associated with NFκB activation, whereas raft disruption switched TNFR signaling from NFκB activation to apoptosis induction (Legler *et al.*, 2003). However, divergent results have been reported for the role of lipid rafts in both TNFR and Fas signaling (reviewed by Muppidi *et al.*, 2004), so the role of lipid rafts in modulating Fas and TNFR signaling is not well understood, and may even be cell-type specific. Nevertheless, further examination of the effects of n-3 PUFA on the activation of these membrane- and perhaps raft-associated death receptors is warranted.

The results of the current research are important for determining how n-3 fatty acids inhibit the growth of breast cancer cells, and how these fatty acids may interact with current cancer therapies. Several agents that are able to inhibit NFκB function are currently in clinical use as cancer chemotherapeutics (Orlowski and Baldwin, 2002) and many more are currently in development (Haefner, 2002). Given that n-3 PUFA alone decrease NFκB activity, it is possible that combining n-3 PUFA with NFκB inhibitors may help to increase their effectiveness or allow for drug dose modification. In addition, activation of Akt has been implicated as one of the mechanisms involved in mammary tumor tamoxifen resistance. Therefore, agents that demonstrate Akt-inhibitory properties may be attractive therapeutic agents or adjuvants for the treatment of breast cancer (deGraffenried *et al.*, 2003). In fact, EPA has been shown to decrease Akt activity and enhance the growth-inhibitory response to tamoxifen in breast cancer cells with constitutively active Akt (deGraffenried *et al.*, 2003), supporting a role for n-3 PUFA as adjuvants to standard anti-cancer therapies. Finally, many tumors remain resistant to treatment with death receptor ligands (eg. TNF, FasL), which is thought to be related to the presence of dominant anti-apoptotic signals (eg. Akt and NFκB) (Fulda and Debatin, 2004). Thus, given the described effects of n-3 PUFA on Akt and NFκB, it is possible that n-3 PUFA may act synergistically with such death-inducing treatments to induce apoptosis in tumor cells. Thus the results of this thesis research demonstrate promise for the use of n-3 PUFA as anti-cancer agents.

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7. APPENDIX

Preliminary *In Vitro* Experiments

Preliminary experiments were conducted to determine optimal growth-inhibitory fatty acid concentrations of EPA and DHA when provided in combination to MCF-7 or MDA-MB-231 human breast cancer cells. LA was chosen as the control treatment as it is an essential fatty acid, it would be constitutively present *in vivo*, and it has been shown to be required for mammary tumorigenesis in the rat (Ip *et al.*, 1985). Treatment with 75 µM LA slightly enhanced the growth of MDA-MB-231 cells (by approximately 10 %) compared to treatment with no fatty acids (Figure A-1) but this was not statistically significant (see also Figure 3-1 in chapter 3). Higher LA concentrations tested (i.e. 100 and 125 µM) did not increase MDA-MB-231 cell growth above that observed with 75 µM LA (Figure A-1). 75 µM LA neither enhanced nor inhibited the growth of MCF-7 cells relative to treatment with no fatty acids (data not shown, but see Figure 3-1 in chapter 3).

The effects of different combinations of EPA and DHA with or without LA on the growth of MCF-7 and MDA-MB-231 cells are shown in Figures A-2 and A-3, respectively.

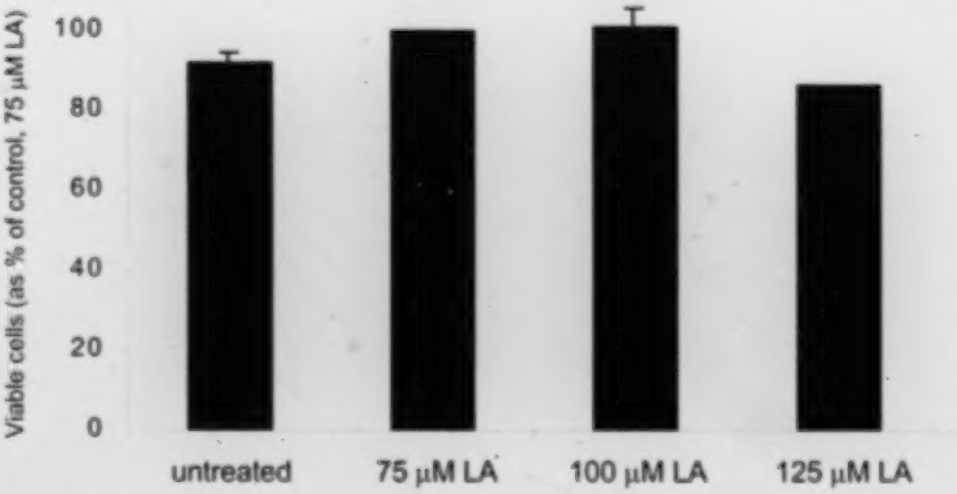

Figure A-1 Viable MDA-MB-231 cells following treatment with LA. Cells were seeded at 1 x 10^6 cells/flask (75 cm^2) in Iscove's modified Dulbecco's medium containing 5 % v/v fetal calf serum and allowed to adhere. After 48 hours, the standard cell culture medium was replaced with fresh medium containing fatty acids (as described in chapter 3) and cells were further incubated for 72 hours. Cells were harvested and viable cells were counted as described in chapter 3. Viable cell counts were normalized to a percentage of the control treatment (75 μM LA), which was taken as 100%. Bars represent the means ± SE of 1 to 5 experiments.

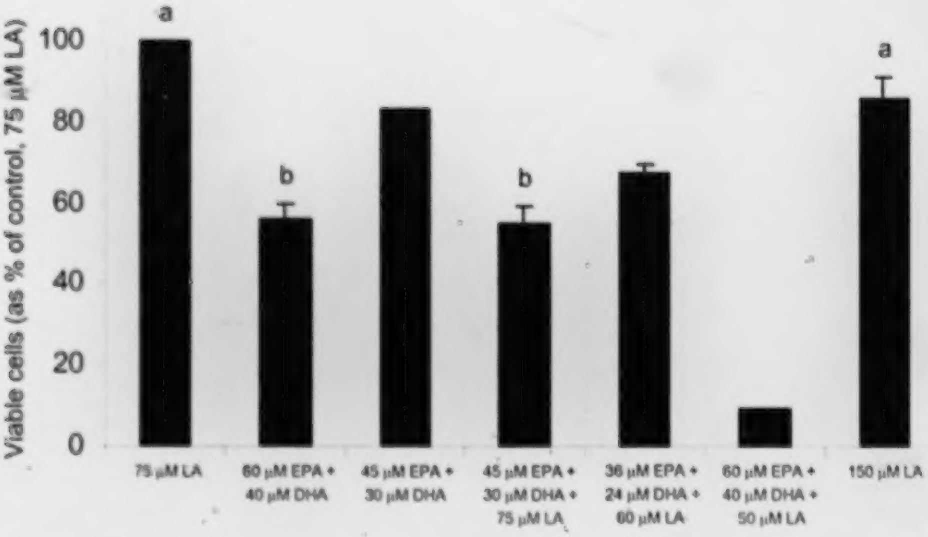

Figure A-2 Viable MCF-7 cells following treatment with mixtures of EPA and DHA. Cells were seeded at 1 x 10^6 cells/flask (75 cm^2) in minimum essential medium containing 5 % v/v fetal calf serum and allowed to adhere. After 48 hours, the standard cell culture medium was replaced with fresh medium containing fatty acids (as described in chapter 3) and cells were further incubated for 72 hours. Cells were harvested and viable cells were counted as described in chapter 3. Viable cell counts were normalized to a percentage of the control treatment (75 μM LA), which was taken as 100%. Bars represent the means ± SE of 1 to 4 experiments. Bars that do not share a letter are significantly different ($p<0.05$). Treatments with no letters had insufficient data for statistical analysis.

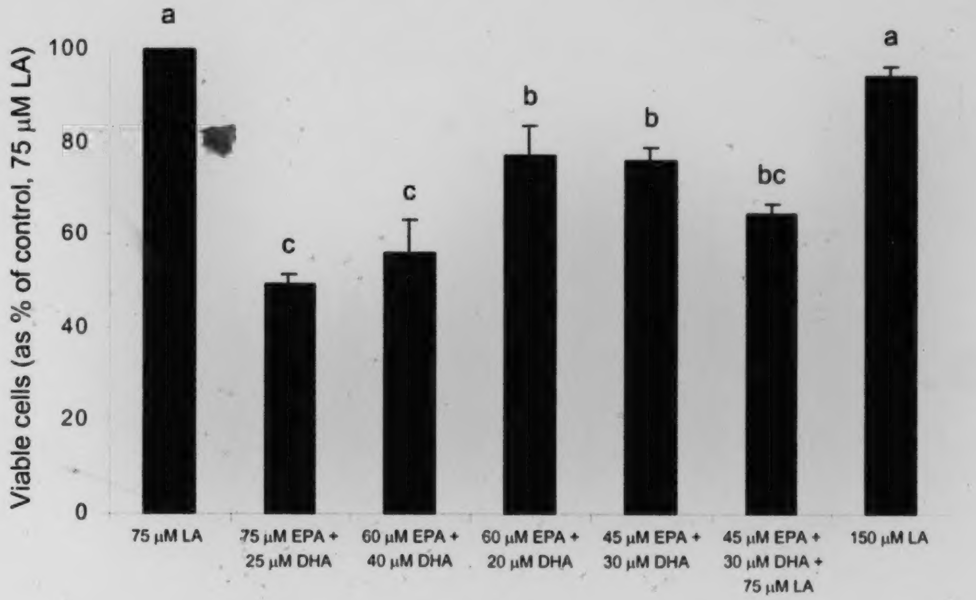

Figure A-3 Viable MDA-MB-231 cells following treatment with mixtures of EPA and DHA. Cells were seeded at 1 x 10^6 cells/flask (75 cm^2) in Iscove's modified Dulbecco's medium containing 5 % v/v fetal calf serum and allowed to adhere. After 48 hours, the standard cell culture medium was replaced with fresh medium containing fatty acids (as described in chapter 3) and cells were further incubated for 72 hours. Cells were harvested and viable cells were counted as described in chapter 3. Viable cell counts were normalized to a percentage of the control treatment (75 μM LA), which was taken as 100%. Bars represent the means ± SE of 4 experiments. Bars that do not share a letter are significantly different ($p<0.05$). (An additional treatment of 60 μM EPA + 40 μM DHA + 50 μM LA was also examined; however cells from this treatment were deemed non-viable after examination under an inverted microscope, and so were not counted.)

These preliminary experiments demonstrated that a mixture of 60 μM EPA + 40 μM DHA significantly inhibited the growth of both cell lines, and to a greater extent than 45 μM EPA + 30 μM DHA or 60 μM EPA + 20 μM DHA. The 60 μM EPA + 40 μM DHA treatment also contains EPA and DHA in a 3:2 ratio which is reflective of the average ratio of EPA to DHA found in most commercially available fish oils used in the animal studies of dietary n-3 PUFA and mammary tumor growth (see Table 1-1 in chapter 1). Therefore, 60 μM EPA + 40 μM DHA was chosen to optimally inhibit the growth of both breast cancer cell lines in the absence of LA. The addition of LA (50 μM) to the 60 μM EPA + 40 μM DHA treatment proved toxic (i.e. the majority of cells were deemed non-viable following examination under an inverted microscope) to both cell types. Thus the EPA and DHA concentrations were titrated down in the presence of LA. The 45 μM EPA + 30 μM DHA + 75 μM LA treatment significantly inhibited the growth of both cell lines, and to a similar extent as the 60 μM EPA + 40 μM DHA treatment. Therefore 45 μM EPA + 30 μM DHA + 75 μM LA was chosen to optimally inhibit the growth of both breast cancer cell lines in the presence of LA. Since we were concerned that higher fatty acid concentrations added to the media might be toxic to the cells, a concentration control treatment was used (150 μM LA) that contained a total fatty acid concentration equal to the EPA + DHA + LA treatment. This 150 μM LA treatment did not significantly decrease the growth of either cell line relative to treatment with 75 μM LA, ensuring that the growth inhibition induced by 45 μM EPA + 30 μM DHA + 75 μM LA was not due to a cytotoxic effect of the total amount of fatty acid in the media. Thus, the four different fatty acid treatments used in this thesis research are: 75 μM LA, 60 μM EPA + 40 μM DHA, 45 μM EPA + 30 μM DHA + 75 μM, and LA150 μM LA.

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